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BIOLOGICAL BULLETIN

OF THE

Marine Biological Laboratory

WOODS HOLE, MASS.

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BIOLOGICAL BULLETIN

THE METABOLIC GRADIENTS OF VERTEBRATE EMBRYOS. III. THE CHICK.

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The aim of these investigations was stated in the preceding papers of this series (Hyman, '21, '26) and repetition here seems unnecessary. Briefly it is hoped that the facts presented in these papers may serve as a basis for the interpretation of normal and teratological development. The method consists in observing the time of death of different regions of the embryo in lethal solutions. The time elapsing between exposure to the solution and death is taken as roughly proportional to metabolic rate and the method thus serves to reveal differences in rate of activity in different parts of the embryo. The grounds on which this interpretion is based are briefly presented in the preceding paper of this series (Hyman, '26) and have been fully discussed in various publications from this laboratory (*e.g.*, Child, '13; Hyman, '20).

The main facts about the gradients of the chick embryo have been known to me for about ten years. The gradients were worked out in detail in 1920 but the data upon the heart were not as complete as desired and publication has been delayed on this account. Every spring and fall since 1923 some time has been spent in studying the disintegration of the heart, a structure not very amenable to investigation by the susceptibility method. Much time and material must be wasted to obtain certain developmental stages which endure but an hour or two. I have finally, however, been able to bring the study to a reasonably satisfactory conclusion.

I. General Disintegration Gradients in the Chick.

In this section of the paper the disintegration gradients of all observable parts of the chick embryo except the heart will be described. The heart will be treated in a separate paper. The observations deal almost wholly with ectodermal and mesodermal structures as entodermal structures cannot be seen clearly enough to enable one to determine the time or course of death in them.

1. Method.—The yolks were poured out into physiological salt solution (0.9 per cent.) warmed to 40° C., and the embryo removed as usual to a watchglass. The salt solution was then withdrawn whereupon the blastoderm with the embryo flattens out upon the bottom of the watchglass. An oval ring, its central opening about the size of the area pellucida, was then cut out of hard filter paper, and lowered on the blastoderm, so that the embryo occupied the center of the opening. By this simple method the blastoderm is held flattened. The watchglass was then filled with the desired killing agent made up in 0.9 per cent. salt solution warmed to 40° C. In most cases the whole was then covered with a thin circular piece of glass with the exclusion of air bubbles. In other cases the watchglass was left uncovered. The death of the embryo was then watched under the low power of the compound microscope. The preparation was kept warm by placing it from time to time on a heated object.

Special care is necessary in removing very young embryonic stages from the yolk, for the blastoderm tears easily and further both the albumen and yolk are strongly adherent to the blastoderm. It was found necessary to proceed as follows in such cases. After emptying the yolk under salt solution, the albumen was completely dissected off leaving the yolk intact. A cut was then made around the blastoderm and by manipulating the cut edge the vitelline membrane was picked up and peeled off leaving the blastoderm still on the yolk. The point of a forceps was then passed beneath the blastoderm freeing it from the yolk. The blastoderm could then be floated off into a watchglass. The blastoderm is freed from adherent yolk by rocking it in several changes of salt solution.

The killing agents used were potassium cyanide, ammonium

hydroxide, and sodium hydroxide. These three reagents were selected because of their high disintegrating powers, probably due to their alkalinity. Owing to the diaphanous nature of the chick embryo, particularly in early stages, the occurrence of disintegration changes cannot be detected unless they are well marked, hence the necessity for agents of the above character. It is also necessary to employ these agents in rather strong concentration so that death will occur in a relatively short time, for the more slowly the death changes take place the less detectable are they. Potassium cyanide was used in about 1/50 mol. concentration, dissolved in 0.9 per cent. salt solution. The ammonium hydroxide was made by adding two or three drops of strong ammonia to 50 to 100 cc. of 0.9 per cent. salt solution. A 1 per cent. solution of sodium hydroxide was prepared in 0.9 per cent. salt and several drops of this were added to the watchglass containing the embryo and half filled with salt solution. In some cases embryos were stained with neutral red before applying the sodium hydroxide; this is a very convenient procedure as the death of any part of the embryo is evidenced by a change of color in the dye from red to yellow.

The results in all three reagents were identical. Potassium cyanide and ammonia are believed to penetrate organisms readily but sodium hydroxide does not penetrate until death has occurred. Death differences cannot therefore be ascribed primarily to differences in rate of penetration of the agents for agents which enter readily and those which do not enter during life kill in the same differential manner. Further, since the agents were made up in salt solution, isotonic with the fluids of the chick, the contention of Wilson ('25) that the disintegration of animals in toxic solutions is due to the hypotonicity of such solutions cannot apply here. The disintegration of chick embryos in such isotonic solutions presents no noticeable differences from the disintegration changes of *Planaria*, for instance, in hypotonic toxic solutions.

Unless specifically stated otherwise several embryos of each stage have been observed. The death gradients in different individuals of the same stage of development are always similar with such variations as are stated in the following descriptions.

The drawings illustrating the death of chick embryos have been simplified and diagrammatized as much as possible. They are made from free-hand sketches drawn while watching the disintegration. Close stippling or solid outlines represent intact structures; open stippling dead and disintegrated structures.

2. Stages before the Appearance of the Typical Primitive Streak.—Technical difficulties have rendered it impossible to reach any satisfactory conclusions concerning very early stages. It is exceedingly difficult, practically impossible, to remove such early blastoderms without inflicting some injury upon them. Since every injury becomes the site of increased susceptibility and since injured places cannot be detected with certainty owing to the delicate texture of the blastoderm, disintegration changes observed in such early blastoderms cannot be regarded as conclusive evidence of innate metabolic differences.

Dr. Leigh Hoadley kindly dissected off several early blastoderms (seven to ten hours incubation) for me but in no case were we convinced that they had been removed uninjured. In such early stages there is generally visible a central area which is slightly more opaque than the remainder of the germinal disk. This opaque area is designated by Hoadley ('26*a*) as the "broad primitive streak"; its appearance is well shown in his figures. It is a somewhat cone-shaped thickening extending from the center to the posterior margin of the blastoderm. In all cases in which disintegration changes were clearly seen in these early blastoderms this opaque region was decidedly more susceptible than the remainder of the blastoderm and there was some evidence that the death changes progressed in it from its anterior to its posterior end.

3. Stage of the Typical Primitive Streak.—This was the earliest stage on which conclusive observations were made. It about corresponds to Fig. 44 *A*, p. 87, of Lillie's text.[1] At this time (about twelve to fifteen hours of incubation), the area pellucida is pear-shaped and the primitive streak is an elongated opaque thickening extending from near or anterior to the center of the blastoderm to its posterior margin. The disintegration of the primitive streak is illustrated in Figs. 1 to 3. Death changes

[1] Lillie, "Development of the Chick," 2d edition, 1919, Henry Holt & Co.

begin at the anterior end of the streak and progress posteriorly, rapidly at first, then more slowly. About seven embryos of this stage were observed and the course of disintegration was identical in all of them.

4. Stage of the Head Process.—This stage is illustrated in Lillie, Fig. 44 *B*, p. 87; its disintegration is shown in my Figs. 4 to 6. Disintegration begins at the anterior tip of the head process and proceeds posteriorly, at first very rapidly, indicating that there is little difference metabolically along the length of the head process, then more slowly. But one blastoderm of this stage was observed although several attempts were made to secure more of them. It seems that this stage endures but a very short time.

5. Stage of the Medullary Plate and the Head Fold.—This stage, illustrated in Figs. 7 to 10, about corresponds to Lillie's Fig. 44 *C*, p. 87. Disintegration begins at the anterior end of the primitive streak, at a point which is probably the primitive knot, and progresses posteriorly along the primitive streak as shown in Figs. 7 to 9. Disintegration is then initiated at the anterior end of the medullary plate and proceeds posteriorly as shown in Figs. 9 and 10.

This stage marks the appearance of the "double gradient" which has been shown to be characteristic of segmented animals (Hyman, '16, '21; Bellamy, '19). There are two regions of high susceptibility, the anterior end of the primitive streak and the anterior end of the neural axis.

6. Early Neural Fold Stage.—The disintegration at this time is similar to the preceding and is illustrated in Figs. 11 to 13.

7. Appearance of the First Somites.—An embryo with one somite and two intersomitic furrows is illustrated in Figs. 14 to 17. It is similar to Lillie's Fig. 50, p. 96. Disintegration begins as before at what I shall call the anterior end of the primitive streak, *i.e.*, the place where the primitive streak is differentiating into notochord and somites. This place presents a club-shaped appearance. From this point disintegration proceeds in both directions, forward involving the somitic region, backward along the streak to its posterior end. Soon the anterior end of the neural folds begins to disintegrate and this progresses posteriorly

along the folds, meeting the disintegration which is advancing forward from the somitic region. The high susceptibility of the lateral boundaries of the embryo is also noticeable.

8. Stage of Three or Four Somites.—The disintegration of a three somite embryo is shown in Figs. 18 to 20. The death changes begin as before at the anterior differentiating end of the primitive streak and proceed in both directions from this region. A region of high susceptibility is now, however, noticeable in the center of the neural folds, as shown in Fig. 18. From this place disintegration proceeds in both directions along the neural folds. The explanation of this circumstance is simple—the neural folds are in process of fusion. As is well known, this fusion does not begin at the anterior end of the neural axis but posterior to this point in a region corresponding according to Lillie, p. 99, "approximately with the region of the future mid-brain or anterior part of the hind brain." There is thus an increased activity preceding the closure of the neural folds for the neural folds meet "by the time four or five somites are formed" (Lillie, p. 98) while the increased susceptibility of this region is already evident at a three somite stage. The somites at this time disintegrate from the segmental plate forward. The lateral boundaries of the embryo are also highly susceptible; processes are evidently in progress there in connection with the separation of the embryo from the blastoderm. The disintegration of four somite embryos is the same as that of three somite stages.

9. Five to Eight Somites.—The region of high susceptibility at the place of closure of the neural folds persists for a varying length of time. Embryos with as many as seven or eight somites may still exhibit traces of it. In such later embryos this region of initial disintegration of the neural tube has moved backward into the hindbrain. Sooner or later, however, this region of high susceptibility in the hindbrain region disappears and the disintegration gradient of the brain region is again of the simple antero-posterior type. Five somite embryos may have already reached this condition. Such an embryo is illustrated in Figs. 21 to 25. Disintegration begins as before in the clavate differentiating region of the primitive streak and proceeds in both

directions, anteriorly along the posterior ends of the neural folds and the segmental plate, posteriorly to the caudal end of the embryo. Disintegration next attacks the anterior end of the neural tube and progresses posteriorly meeting the other disintegration at the level of the first pair of somites. In another embryo of five somites, the region of high susceptibility in the hindbrain, correlated with the closure of the neural folds, was still present. The disintegration of the neural tube of this embryo is shown in Figs. 26 and 27; otherwise it was identical with the embryo represented in Figs. 21 to 25.

The disintegration of an embryo of seven somites, corresponding to Lillie's Fig. 59, p. 104, is illustrated in Figs. 28 to 30. Death changes begin as always in the clavate differentiating region of the primitive streak and proceed anteriorly from here along the segmental plate and posterior ends of the neural folds. They then begin in the fore-brain, in the optic evaginations, and progress posteriorly along the neural axis. Other embryos of seven and eight somites may still exhibit barely perceptible traces of heightened susceptibility in the region where the neural folds are closing.

In all embryos up to seven or eight somites the gradient in the somites is, as far as could be determined with certainty, from the segmental plate forward. It is somewhat difficult to observe the somites in these early stages owing to the fact that the neural folds as they die spread open and conceal the somites from view. In a number of cases, however, the death of the somites was observed with certainty and is, as stated, from the segmental plate anteriorly.

10. Nine to Eleven Somites.—The chief point of interest about embryos of these ages is the rapidly increasing susceptibility of the optic evaginations. The death of an embryo of eleven somites is illustrated in Figs. 31 to 35. Disintegration begins in the anterior end of the primitive streak, as usual, and simultaneously in the tips of the optic vesicles. From the first named region it progresses anteriorly along the neural folds and segmental plate. The optic vesicles disintegrate from their lateral extremities medially; disintegration then passes posteriorly along the neural tube meeting the advancing postero-

anterior disintegration at about the middle of the somites. From this time on a double gradient is present in the somites; anterior and posterior somites are the most susceptible and from them death proceeds towards the middle somites.

11. Stage of Twelve Somites.—In this, the stage of the classical 33-hour chick (Lillie, Fig. 63, p. 109), a new region of high susceptibility makes its appearance, at the anterior end of the hind brain. The disintegration of a twelve somite chick is represented in Figs. 36 to 39. The high susceptibility of the optic vesicles has now died away; evidently the process of evagination is completed. Disintegration begins in the anterior end of the primitive streak and the anterior end of the neural axis and proceeds anteriorly from the former, caudally from the latter. Very soon, however, the region in the hind brain mentioned above begins to disintegrate and from this region disintegration progresses in both directions along the neural tube as shown in Fig. 37. This occurrence, at first puzzling, was readily interpreted after later stages had been studied. It was then realized that this region of high activity in the hind brain of the twelve somite chick foreshadows physiologically the process of turning of the head of the embryo which is not manifested morphologically until a slightly later stage.

12. Fourteen to Twenty Somites.—The head of the embryo now begins to turn on its left side and the stalks of the optic vesicles narrow. Both of these processes are indicated in the disintegration. The disintegration of a fifteen somite chick is shown in Figs. 40 to 44. Death begins in the anterior end of the primitive streak and in the stalks of the optic vesicles. From the former it progresses forwards along the neural folds and segmental plate; from the latter laterally to the tips of the vesicles. Disintegration next occurs at the anterior end of the forebrain and in the hind brain; from these two regions it progresses backward and forward respectively, meeting in the region of the midbrain. The high susceptibility of the hindbrain is evidently correlated with the turning of the head; it should further be noted that the region of the brain involved in this process has moved posteriorly as compared with the preceding stage. In the remainder of the body disintegration proceeds

along the neural tube and somites from the two ends of the body towards the middle, the two waves of disintegration meeting at the middle of the somites.

From this time on through more than twenty somites the disintegration gradients are about the same. The turning of the head as well as the advancing head fold of the amnion obscure observations on the disintegration of the head. The region of high susceptibility in the hindbrain becomes more marked and involves a larger area. The advancing margin of the head fold of the amnion and also the auditory pits are regions of high susceptibility.

13. Two-Day Chick.—This stage, illustrated in Figs. 45 to 47 is marked by the high susceptibility of the eye. The formation of the lens and the optic invagination are at their height. The eye and the posterior end of the embryo are the most susceptible parts. After the eye has disintegrated, disintegration begins in the tip of the telencephalon and proceeds posteriorly along the brain. A region of high susceptibility is also still present at the place where the body is turning and from this place disintegration progresses in both directions. The auditory vesicles disintegrate before the hindbrain. There is the usual postero-anterior progress of the disintegration from the primitive streak.

14. Two- to Three-Day Chicks.—In later stages the region of high susceptibility at the bend of the embryo gradually disappears; it can still be detected at the beginning of the third day of incubation but subsequently vanishes. The susceptibility of the posterior end of the embryo also gradually diminishes. In the two-day chick the tail bud is still the most susceptible part of the embryo, but in the three-day chick, the tail bud does not begin to disintegrate until sometime after anterior structures have undergone disintegration. Of anterior structures the eye is by far the most susceptible in these later stages. Disintegration begins in the lens vesicle and then extends to the optic cup. Next the telencephalon disintegrates and this process proceeds posteriorly along the brain. The gradient of the brain is rather shallow, however. At all stages which were observed the double gradient was present but the susceptibility of the posterior end as compared with the anterior end diminishes in the later stages,

as already mentioned. After disintegration has begun at the posterior end it proceeds forwards, in the neural tube first, then in the somites; the last region of the embryo to disintegrate is in general at about the level of the middle somites. The anterior somites are commonly more susceptible than the posterior ones and susceptibility in the somites decreases from both ends towards the middle. The limb buds in the three-day chick are regions of moderately high susceptibility, the anterior limb buds being more susceptible than the posterior ones. The ear vesicles are more susceptible than the adjacent region of the brain in the two-day chick but their susceptibility is decreased in the three-day chick. The visceral arches in all stages in which they could be observed with certainty were the least susceptible parts of the embryo; they appeared to disintegrate from the posterior end forwards as do the somites in early stages.

In these later stages of the chick evidences of a medio-lateral gradient were noted. If we consider a section through the middle of the body, the neural tube is the most susceptible part of the section; the medial parts of the somites disintegrate next; and the disintegration then proceeds laterally along the somites to and along the lateral plate. In two- and three-day chicks where the first somites present their lateral surfaces to the observer, owing to the turning of the trunk, it could easily be seen that the medial portions of the somites are more susceptible than their lateral portions.

15. Entodermal Structures.—The posterior part of the notochord can usually be seen more or less distinctly. This part disintegrates from the primitive streak anteriorly. The anterior part of the notochord is more or less obscured but appears to disintegrate from the diencephalon caudally. The gradient in the notochord is thus probably of the double type as in the later somites and the neural tube.

If the embryo is mounted ventral side up, the ventral layer of the blastoderm, which is of course composed of entoderm, is exposed to view but is of so delicate a texture that its time of disintegration is difficult to determine. In the posterior part of embryos up to two days of age the entoderm layer also appears to disintegrate from the posterior end forward, thus accompanying

the disintegration of the neural tube. The entodermal layer of the anterior intestinal portal is always a region of high susceptibility and from the portal the disintegration extends anteriorly along the entoderm of the blastoderm. The foregut could not be distinctly observed.

16. Relative Susceptibility at Different Ages.—While this matter was not subjected to definite experimental test, the much greater susceptibility of the younger stages was so obvious that it could not escape attention. A primitive streak stage will disintegrate in a few minutes in a concentration of reagent in which a two-day chick will not undergo complete disintegration in two hours.

17. General Summary of the Gradients in Chick Embryos.—In the early stages—primitive streak and head fold stages—a simple primary antero-posterior gradient is present, disintegration beginning at the anterior end of the embryonic axis and proceeding posteriorly. At the time of the differentiation of the medullary plate and folds a double gradient appears and persists through all of the stages that were investigated (through the third day). In general this double gradient is as follows: there are two regions of high susceptibility, the differentiating region of the primitive streak, and the anterior end of the neural axis; from these two regions disintegration proceeds anteriorly and posteriorly, respectively, towards the middle of the embryo. The differentiating region of the primitive streak is the most susceptible part of the embryo up to about the third day of incubation at which time it gradually decreases in activity in correlation with the completion of the somites. The double gradient involves both ectodermal and mesodermal structures; and probably also to at least some extent, entodermal structures. In earlier stages disintegration in the mesoderm proceeds from the segmental plate forward along the somites; but after eight or nine somites have appeared, the anterior and posterior somites are both regions of high susceptibility and from them disintegration proceeds backward and forward respectively towards the middle. The neural tube is usually more susceptible than the mesoderm of the same level. There is also present a mediolateral gradient in susceptibility from the neural tube towards the lateral plate.

In addition to this general double gradient, special regions of high susceptibility appear temporarily during development. The most important of these are: all places where folds, unions, or bendings are about to occur; and the special sense organs.

In general any extensive developmental change is preceded by an increased susceptibility, that is, an increased metabolic rate in the group of cells involved. Intense metabolism thus appears to be a necessary condition for active development.

II. Application to Normal Development.

The foregoing observations may throw some light on problems of amniote development and on the physiological conditions underlying normal and teratological development. In utilizing these observations for the interpretation of development, the susceptibility differences described in the preceding section will be regarded as representing differences in the rate of cell activity, the most active regions being the most susceptible to lethal solutions.

The primitive streak is described in textbooks of embryology as an undifferentiated linear ectodermal thickening. The important researches of Hoadley ('26, *a*, *b*, *c*), however, have shown that there is some localization of developmental potentialities in blastoderms younger than the typical primitive streak stage, blastoderms of four to eight hours incubation. My observations on the typical primitive streak stage (about fifteen hours incubation) indicate that there is also present a physiological gradation along the streak. The anterior end of the streak is the seat of a high rate of activity, this rate diminishing posteriorly along the streak. My observations of course do not show that this physiological differentiation precedes the morphological localization for the investigations of Hoadley concern earlier stages than those on which I was able to observe reliable susceptibility differences. But it must be recalled that in the hen's egg the direction and position of the primary antero-posterior axis of the future chick are already established in the ovocyte, being determined by the attachment of the follicle in the ovary. In the pigeon's egg also Bartelmez ('12) showed that the main axis is determined in the ovocyte and is evidenced during the

blastodisc stage through the position of certain granules. As Hoadley found a lack of localization of future structures in unincubated blastoderms or those incubated two hours it appears that it must be conceded that some physiological differences underlie the subsequent localization of organs along the axis.

The nature of the head process has been a subject of discussion. My observations indicate that the head process stage does not differ as regards metabolic conditions from the primitive streak stage and thus support the conclusion of Lillie (p. 82) that the head process is simply a further development of the anterior end of the primitive streak. The region of high metabolic activity at the anterior end of the streak develops into the head process.

In subsequent development this region retains its high rate of activity and becomes the anterior end of the neural axis. But at about the time of origin of the medullary folds, a secondary region of still higher activity appears. This region is located at the anterior end of the primitive streak (excluding the head process as part of the streak) or in what is known in textbooks of embryology as the primitive knot or Hensen's node. The origin of such a second region of intense activity when one such region is already present can from a physiological point of view be ascribed only to the inability of the latter to control more than a short length of the axis. Beyond that region of control physiological isolation occurs and the origin of a new center of activity is possible. This new center being isolated from the dominance of the original center at the anterior end of the embryonic axis behaves as isolated regions always do when they are intrinsically capable of such behavior—it proceeds to the formation of new individuals, *i.e.*, segments. The experiments of Bellamy ('19) on the frog constitute the best evidence that we have of the applicability of this interpretation, originally derived by Child from experiments on lower invertebrates, to the vertebrate embryo. Bellamy showed that in the frog embryo the distance between the two centers of high activity can be altered by the action of external factors. As the anterior center of activity of the chick embryo differentiates into nervous tissue it is able to dominate more of the axis; the secondary active region, localized in the anterior end of the primitive streak

thereupon moves backwards, or more strictly speaking is transferred to more and more posterior cells, leaving incomplete zooids or segments anterior to it. Finally the anterior end dominates the posterior center of activity to such an extent that it is no longer able to carry on independent processes and segment formation thereupon ceases.

How much of the embryo arises from the primitive streak is a question of considerable interest to embryologists. The answer to the question will of course depend on what is meant by primitive streak. If following the usual usage we regard the primitive streak as that portion of the early embryonic axis including and posterior to the secondary center of activity, that is, the primitive knot, the answer can be given on *a priori* grounds from the argument in the preceding paragraph as follows: the primitive streak gives rise to all of that part of the embryo which is segmented, that is, from the first somite posteriorly. This conclusion agrees with the experimental results of Peebles ('98).

One of the most striking facts that has appeared in this investigation is the high susceptibility of each region where development is progressing rapidly. This indicates that intense metabolism is a necessary condition for active development. According to my observations such developmental processes as the closure of the neural folds, elevation of the amniotic folds, and formation of the body and brain flexures are the result primarily of cell activity and not a consequence of mechanical conditions as supposed by early embryologists. It further appears from my observations that intense cellular activity precedes such developmental processes. This was clearly the case for instance as regards the closure of the medullary folds and the turning of the embryo. The increased activity in these processes is evidenced before the morphological change is initiated.

From my studies of the metabolic gradients in the embryos of the teleosts ('21), the brook lamprey ('26) and the chick and the work of Bellamy on the frog ('19) it seems justifiable to conclude that the formation of two centers of high activity is the regular mode of development of vertebrates. Two such centers have been found in all vertebrate embryos so far studied and they are always located in the same position with respect to the future

embryo—one at the anterior end of the antero-posterior axis and the other in the axis at a more posterior point. The posterior center is the dorsal lip of the blastopore in cyclostome and amphibian embryos (probably also in amphioxus), the posterior end of the embryonic axis in teleost fish, and the primitive knot, subsequently the "tail bud," in the chick. This posterior center of activity is like a growing point which growing backwards deposits the trunk of the embryo anterior to it. It is interesting to note that Assheton ('94*a*, '94*b*) long ago recognized in the embryos of the frog and the rabbit the presence of these two centers of activity and correctly identified the posterior one as the dorsal lip of the blastopore in the frog and a region about corresponding to the primitive knot of birds in the rabbit. He called these regions primary and secondary centers of cell proliferation. He stated that the primary center forms the head of the embryo anterior to the first somite, while the secondary center forms the rest of the embryo. My ideas here presented entirely coincide with these statements of Assheton, which seem to have been generally overlooked. Eycleshymer ('98) working with the amphibian embryo accepted Assheton's ideas. He showed that the primary center, the center of the animal pole, becomes the anterior end of the embryo, the dorsal lip of the blastopore the trunk. He considers these to be two regions of high activity, agreeing with Assheton. He stated: "The primary area of cell activity at the upper pole of the amphibian egg forms the basis of the cephalic end of the embryo." "The secondary area of cell activity on the blastoporic side of the egg forms the basis of the greater portion of the posterior half of the embryo." (Eycleshymer entertained the probably erroneous notion that the extreme posterior end of the amphibian embryo was formed by concrescence.) Adelmann ('22) and Kingsbury ('24) seem to agree with the conception that the formation of two centers of high activity is the regular mode of development among chordates. Adelmann's idea that these two centers arise by the splitting of one center is, I think, incorrect. It does not agree with the actual facts of observation by the susceptibility method on several types of vertebrate embryos. The posterior center arises independently of and usually at some distance from the

anterior center, although in the chick the two centers are somewhat close together at first. In all cases a region previously lower in susceptibility than the anterior center begins to increase in susceptibility and generally eventually surpasses the anterior center in activity for some time. Further criticisms by Adelmann and Kingsbury of the application of susceptibility data to chordate development have been answered by Child ('25).

I also wish once more to record my disbelief in the concrescence theory of the formation of the primitive streak. I find no evidence in any of my work with vertebrate embryos of the occurrence of such a process. Neither can I accept the germ ring interpretation of chordate development as expounded for instance in Kellicott's "Chordate Development." The germ ring type of development occurs in certain meroblastic eggs and appears to be simply a method of inclosing the yolk by the blastoderm. The germ ring of the teleost embryo does not appear to be of much more importance in the formation of the embryo than the growing margin of the blastoderm in the chick embryo. The posterior growing point of teleost embryos situated in the germ ring corresponds in my opinion to the dorsal lip of the blastopore of amphibian embryos. It probably appropriates neighboring cells as does also the latter.

Textbooks of vertebrate embryology should probably be revised, emphasizing the origin of the chordate embryo from two centers of activity, a primary center which forms the anterior end of the embryo and a secondary posterior center which grows backwards and forms the remainder of the embryo. The recent beautiful experiments of Spemann and Mangold ('24) establish beyond doubt the importance of this secondary center in the production of the embryo. Whether or not a primitive streak appears in the development of a chordate seems to depend on the time relation between gastrulation and the establishment of the posterior center. If the posterior center arises somewhat tardily after gastrulation development occurs with the formation of a primitive streak; but where the center is early established a primitive streak is absent from the development since differentiation then begins at once.

Faris ('24) in a study of pigment formation in the *Amblystoma*

embryo has expressed the opinion that it is necessary to distinguish between differentiation metabolism and proliferation metabolism. The former has probably a greater integrative action than the latter. This idea may be very important in the interpretation of developmental processes but cannot be evaluated at present.

The question of the origin of metabolic differences in embryonic development is naturally of prime importance but little evidence is as yet available upon this matter. In the case of the hen's egg it seems definitely established that the axis of the embryo is determined by the position of the ovocyte in the ovary, *i.e.*, by an environmental factor acting in a definite direction. Similarly it may be supposed that the metabolic conditions present at each developmental stage are determined by factors operative at preceding stages. In the last analysis environment must be the cause of metabolic differences.

III. Application to Teratological Development.

Abnormal chick embryos accidentally or experimentally produced have long been of interest to zoölogists. Many articles have been published dealing with the experimental production of abnormalities in the chick but most of them are rather old and I have not been able to obtain access to a number of them. Others give such inadequate accounts of the abnormalities found that they are of little value from the present point of view.

The interpretation of experimentally produced abnormalities in the chick on the basis of differential susceptibility will naturally follow the same lines as stated in previous publications by Child and his students on developmental modifications in other animals. Those portions of the embryo which are the most susceptible to lethal concentrations of toxic agents will be most affected and modified by non-lethal exposures of proper duration and concentration in which acclimation does not occur. These same portions will recover most readily if the concentration and duration of exposure employed permit recovery and acclimation. If the metabolic rate of the egg or early embryo is permanently depressed and slowed down by the action of external agents, parts requiring for their normal development a high metabolic

rate will be completely suppressed or will be abnormal because the rate of activity necessary for their normal development cannot be attained in such depressed eggs or embryos. The various agents which can be used to affect development are regarded from this viewpoint as acting merely in a quantitative way to retard or accelerate development; and this action is differential affecting some parts of the embryo more than others since some parts have or require a higher rate of activity than other parts.

Interpretations of teratological development somewhat similar to the foregoing have not been wanting. Dareste ('91) who investigated more thoroughly than anyone else before or since the experimental production of monstrosities in the chick expressed similar ideas. He states that development is due in part to inherent tendencies and in part to external conditions. As the latter can be altered, modification of the course of development is possible. Dareste reached the following important general conclusions. (1) The same abnormalities are produced by very different conditions, there being then no necessary relation between the application of a certain condition and the appearance of certain modifications. (2) Embryos submitted to the action of identical factors do not necessarily present the same abnormalities (although these may often be similar). The reason for this is that eggs are inherently different from each other in a variety of ways. (3) The different abnormalities do not depend on the nature itself of the teratogenic agents but on the time at which they act upon the embryo, their intensity, and their duration. (4) All abnormalities consist essentially of an arrest of development of the embryo or its membranes. The organs of an embryo appear successively; they pass through a certain number of stages. An arrest of development consists in the persistence of an embryonic state ordinarily transitory. Among the abnormalities assigned by Dareste to arrest of development at a certain stage are: duality of the heart, spina bifida, absence or poor development of the area vasculosa, absence or abnormalities of the amnion and allantois, defects of the brain, head, spinal cord, and sense organs. Certain subsequent interpretations of teratological development as for instance that of Stockard ('21) do not appear to differ from or add anything to Dareste's conclusions.

The work on differential susceptibility and modification of development carried on in this laboratory by Child and his students supports Dareste's fundamental conclusions but has added one important factor to the interpretation of teratological forms. The statement of Dareste that most abnormalities are the result of retarded or arrested development is correct but needs to be carried one step farther. *Inhibition or retardation modifies the development of some parts of the embryo more than of others,* so that *certain* regions *frequently* exhibit teratological development while other regions *seldom* do so. Those regions most prone to abnormal development under the action of inhibiting agents of proper concentration and duration of exposure are those which have been shown by Child and his students to be most susceptible and most quickly killed by lethal concentrations of these agents. In brief differential inhibition of development results from differences in susceptibility of different parts of the embryo.

On the basis of the susceptibility differences described in this paper the application of external agents of a depressing nature to the chick embryo should yield the following results. Agents applied at very early stages may produce a permanent general diminution of the metabolic rate of the embryo. Such a condition will result in the absence or abnormality of those parts of the embryo requiring a high metabolic rate for their normal development, for in such retarded eggs the necessary rate of activity cannot be attained. Sufficiently drastic treatment at an early age will prevent the appearance of the embryo altogether, only the blastoderm being present; for as shown in this paper the embryonic is more susceptible than the extra-embryonic portion of the germinal disk. Less drastic treatment of early embryos up through the head process stage (fifteen to eighteen hours of incubation) should cause defects of the anterior end mainly, particularly the brain, since during this period of development there is but one center of high activity, the anterior end of the embryonic axis. Exposure to depressing agents during later stages of incubation (medullary fold through the two-day chick stage) would be expected primarily to stop or retard the posterior elongation and in addition to evoke abnormalities of the anterior

end and special sense organs; for during this time there are two centers of high susceptibility, the anterior end of the embryo and the growing region of the primitive streak. Application of depressing agents at the proper times when such processes are occurring would tend to prevent the occurrence of unions, and the formation of folds, bends, turns, etc., and would induce abnormalities in particular structures, such as the sense organs and limb buds. It is not, however, necessary in the case of structures requiring a very high rate of activity for their normal development that the inhibiting agents be applied at the time they are developing (contrary to the opinion of Stockard, '21); for any general lowering of the metabolism of an embryo at an early stage hinders the future development of such structures. Thus it has been shown that the usual typical abnormalities appear when the egg is subjected to depressing agents before fertilization or when the sperm are injured (for literature on this matter see Hyman, '21). Probably structures which develop at a low metabolic level cannot be inhibited or altered unless depressing agents are applied at the time of their greatest developmental activity.

As noted long ago by Dareste, abnormalities can be induced in the chick by a variety of methods and such abnormalities bear no specific relation to the agent employed. I shall here review the different agents which have been used and the abnormalities obtained by their action, considering only single monsters.

Dareste ('83*b*, '91, p. 103 ff.) found that shaking eggs induces abnormalities of development. Eggs incubated immediately after a railroad journey are apt to develop abnormally but yield normal embryos if several days elapse between the journey and incubation. Eggs shaken violently on a machine yield a high percentage of abnormalities whether incubated immediately or some time after being shaken. In about half of the eggs so treated blastoderms without embryos were found. When embryos were present these were generally abnormal, exhibiting such conditions as doubled hearts, defective eyes, reduced size, defective brains, spina bifida, and absence of the posterior end.

According to Dareste and others (Dareste, '91, pp. 161, 168),

eggs tend to develop abnormally if they are not turned during incubation or if they are incubated in the vertical position. Few details are, however, furnished as to the types of abnormalities so induced. They are stated to result from the adherence of the embryonic membranes to the shell.

Several workers have reported induction of teratological development in the hen's egg through exposure of the eggs to the electric current or a magnetic field (Lombardini, '68, Maggiorani, '84, Windle, '91, '93, '95). I have not been able to obtain these publications except two of Windle's but it appears that very few details were given in them of the types of abnormalities obtained. Probably the teratological action really resulted from the products of electrolysis rather than directly from the current or magnetic fields. Windle found a high percentage of malformed embryos with defective areæ vasculosæ but Maggiorani reported an excessive vascularization of the yolk sac with arrested development as a result of incubating eggs between two powerful magnets.

There has been some investigation of the action of X-rays and radium emanations on the development of the chick. Gilman and Baetjer ('04) exposed hen's eggs to X-rays ten minutes a day for four successive days and noted certain abnormalities—deformed occipital region, retarded eyes, membranes adherent to the embryos, and deformations of the limbs. Tur ('04) subjected hen's eggs continuously to radium placed on the outside of the shell during periods of incubation varying from 24 to 70 hours. After 24–28 hours of such treatment, blastoderms of normal size were found, but the area pellucida was reduced, and only the posterior part of the primitive streak was in evidence, the anterior part having been reduced to an irregular mass. After 45 to 70 hours of incubation under radium emanation blastoderms without visible embryos were obtained. In some cases the area vasculosa was present and more or less normal in appearance; in other blood and blood vessels were lacking and the area pellucida was greatly reduced. Sections of such blastoderms in the region where the embryo was to be expected revealed the presence of a flattened inactive ectoderm and a thickened proliferating entoderm showing numerous mitotic figures. Tur's

results agree with my findings that the embryonic is more susceptible than the extra-embryonic portion of the blastoderm and the anterior part of the primitive streak more susceptible than its posterior part.

More numerous have been the attempts to modify the development of the chick by various chemicals. Féré ('94, '99*a*) exposed eggs to the vapors of ethyl and other alcohols before incubation, and reported the following abnormalities: blastoderms without embryos, arrest of development of the amnion, cyclopia, absence of the eyes, defective heads, atrophy of the head, spina bifida, absence of somites, duplicity of the heart. He also ('99*b*) tried the effect of exposure to the vapors of ammonia before incubation. Ammonia was found to be exceedingly toxic; one half hour exposure yielded blastoderms with embryos. After fifteen minutes exposure, defects similar to those obtained with alcohol resulted. Reese ('12) tried the effect of various narcotics on the development of the hen's egg but gives no details beyond the statement that abnormalities were produced. Stockard ('14) treated the egg with various chemicals and states as did Dareste that their action is not specific. Eggs were exposed to the vapors of alcohol and ether before or during incubation. The treated embryos tended to be small and developed more slowly than the controls. Among the defects noted were: blastoderms without embryos, small and reduced embryos with defective brains and circulatory systems, one or both eyes defective, cyclopia.

Alteration of the oxygen supply to the egg is a commonly used method of inducing teratological development. The earlier attempts in this direction consisted in covering the whole or portions of the egg with a varnish. The original purpose of such experiments seems to have been to prove the necessity of oxygen for the development of the chick. If the entire egg is varnished the embryo of course dies at a certain stage of development as shown long ago by Dareste. If the egg is partially varnished, development tends to be abnormal. Gerlach and Koch ('83) varnished the entire egg except for a circle 4.5 to 6 mm. in diameter immediately over the blastoderm. The general consequence of such procedure was a dwarfing, often extensive, of the embryo, due in my opinion to inhibition of the growing region of

the primitive streak. Similar dwarfing accompanied by other abnormalities resulted when the unvarnished circle was over the anterior or posterior part of the blastoderm. Windle ('93) applied varnish to different parts of the egg. Among the abnormalities regularly obtained were: defects of the area vasculosa, absence of the embryo, deformed embryos. Here again the account is too lacking in detail for interpretation. Mitrophanow ('00) also tried the effect of partial shellacking of the egg, combining this procedure, however, with heightened temperature. Upon shellacking that half of the egg containing the anterior half of the embryo and incubating at supernormal temperature (about 45° C.) Mitrophanow noted a general inhibition of development of the anterior part of the primitive streak and of the head process with broadened and thickened posterior ends. Mitrophanow regards such posterior ends as overdeveloped but it seems to me that they may represent inhibitions. It appears that if the posterior center of activity is somewhat inhibited it tends to pile up into masses of cells instead of elongating in the posterior direction. In one case, which was in the early medullary fold stage when opened, the primitive knot region was found greatly inhibited, regions anterior and posterior to this being more or less normal. Shellacking the posterior half of the egg did not yield any definite modifications of development except some inhibition of the posterior end. Mitrophanow was of the general opinion that the posterior end of the early embryo is most susceptible to modification. The most extensive experiment on the production of abnormalities in birds by an altered gaseous environment is that of Riddle ('23). Unfortunately the data furnished by Riddle as to the modifications induced are very meager. Further the embryos were examined mostly at late stages or at hatching and many of the most abnormal embryos were thus probably missed, having died at early stages. Riddle records several dove and pigeon embryos with defective or absent eyes, small heads, and other head defects as a result of exposure to air reduced in oxygen content (11 to 13 per cent. oxygen) during about 24 hours in the first three days of incubation. Excess oxygen with or without increased carbon dioxide content yielded similar abnormalities. Riddle states that nearly

all of the abnormalities arose from embryos "aged less than two days at the time treatment was begun." Byerly ('26) allowed eggs to incubate for 24 hours and then coated them with water-glass after removing the entire shell and outer shell membrane from the air chamber. Such embryos were then incubated for three days. Some of the embryos thus obtained were very abnormal, the posterior end being most affected. This agrees with my finding that at 24 hours the posterior end of the embryo is more susceptible than any other part.

Although the modifications induced by these various procedures agree with the expectations on the basis of differential susceptibility, nevertheless the applicability of this interpretation in these cases may be open to question: for there is no certainty that all parts of the embryos were equally exposed to these agents. Variations in different regions of an egg as regards thickness and porosity of the shell, viscosity and thickness of the albumen layer, permeability of the shell and egg membranes, and similar factors render it highly probable that external agents and conditions will not reach all parts of the embryo simultaneously. To mention only one condition, an altered gaseous environment obviously requires some time to affect the embryo. Obviously the principle of differential susceptibility will apply to the induced abnormalities with certainty only when the conditions or agents applied have reached all parts of the embryo equally and simultaneously. Apparently the only conditions under which these prerequisites are fulfilled are ageing of the eggs and alterations of the temperature of incubation.

Dareste ('82, '83*a*) found that if eggs are kept for some time before being submitted to incubation, development is abnormal. Typical abnormalities were: blastoderms without embryos, embryos with absent or reduced posterior ends, reduced and abnormal heads and brains, and celosomia (protrusion of the viscera due to a failure of the lateral limiting sulci to close below). It is possible of course that a beginning slow development at room temperature as well as senescence of the embryo is a factor in these cases.

A number of experimenters have tested the effect of subnormal or supernormal incubation temperatures on the development of

the chick. Dareste regarded alteration of temperature as one of the best methods of inducing teratological development in the chick. According to Dareste, the hen's egg will not develop below 28° C. but Edwards ('02) places the "developmental zero" at 20°–21° C. Dareste found that the embryo dies at 44° or above but Mitrophanow ('00) would seem to have obtained development at temperatures up to 50. According to Dareste development below 35° or above 39° C. is nearly always abnormal.

Féré ('94) in eggs incubated at 40° and 41° C. noted blastoderms without embryos, defective heads, defective optic vesicles, spina bifida, failure of the somites to form. Kaestner ('95) performed an extensive series of experiments in which eggs after being incubated at normal temperature for various lengths of time were exposed to low temperatures for various periods, and then returned to normal incubation temperature. He found that when such developing eggs are kept too long at the lowered temperature, the time varying with the age of the embryo when exposed, abnormalities were obtained. They occurred most frequently when the temperature was lowered during the first two days of incubation. Disturbances of the head, heart, and brain were noted, and inhibition of such processes as the closure of the medullary folds, elevation of the head fold of the amnion, and union of the heart anlagen. Alsop ('19) incubated eggs at subnormal and supernormal temperatures and examined the embryos after one to three days incubation. At subnormal temperatures (94° to 102°), she noted failure of the neural folds to close, thickenings at the primitive knot, abnormal neural tubes often blocked by thickenings particularly in the lumbar region, curvature of the primitive streak. It seems probable to me from inspection of Alsop's figures that the thickenings noted are due to inhibition of the growing point of the primitive streak. At supernormal temperatures (104° to 108° C.) abnormalities of the brain, particularly of the optic vesicles and midbrain, were most frequent. Alsop's figures, however, also show inhibition in the primitive streak. In some cases extra somites appeared. Riddle ('23) exposed dove and pigeon embryos to subnormal temperatures for various periods during the first days of development but noted only a small percentage of

abnormalities. I may here report a number of abnormal chicks obtained from eggs unintentionally left in the incubator room for several days before being placed in the incubator. The temperature of this room is about 30° C., a temperature at which, as is well known, the hen's egg will develop slowly for some time. These eggs were incubated about thirty hours: all of the chicks alive at the end of this period, about one dozen, were abnormal. The growing region of the primitive streak was in all cases inhibited to a greater or less extent. In most of the embryos it formed one or more irregular projecting masses of cells. As a result of this inhibition of the primitive streak, the posterior half of these embryos is too short and with too few somites as compared with the anterior half. One of these embryos is illustrated in Fig. 48. The anterior half of this embryo is normal (except the heart) but posteriorly the primitive streak terminates in a large projecting mass. On the right side the number of somites is about normal for the state of development of the anterior end but on the left side the number is considerably reduced. The posterior end of the neural tube is inhibited: the tube is widely open and the neural folds poorly developed especially on the right side. As already stated, eleven other embryos with similar conditions of the posterior end were obtained. Of these twelve embryos the anterior end appears entirely normal except in two cases where the head is exceedingly abnormal represented only by a crumpled mass at the level of the heart, the heart being then located anterior to this mass. This condition was called omphalocephaly by Dareste. One of the two embryos of this type obtained is illustrated in Fig. 49. The posterior end is nearly normal but the greater part of the head is invaginated into a mass containing crumpled fragments of neural tube. This mass is situated to the left of the embryo. The heart is at the anterior end. In the majority of these embryos the heart is abnormal.

The most complete description of abnormalities in the chick is that of Dareste in his well-known book. As Dareste found that all experimental procedures induce practically the same types of anomalies, he does not state in his description how the different abnormalities were produced. He lists the following

as the chief abnormalities observed by him in the chick embryo. (1) Duality and other anomalies of the heart. Dareste correctly ascribed the double heart to an inhibition of the union of the two heart tubes. (2) Blastoderms without embryos. In these cases the blastoderm consists only of ectoderm and entoderm, mesoderm being absent. (3) Anomalies of the area vasculosa, absence of blood islands and blood vessels. (4) Anomalies of the amnion and the allantois, due to inhibition at the time when these membranes are forming. Dareste thought many other abnormalities were caused by the suppression of the amnion, but he was probably incorrect in this opinion. (5) Asymmetries of various kinds, as of the eyes and two sides of the head. (6) Spina bifida, correctly ascribed by Dareste to inhibition of the closure of the neural folds. (7) Omphalocephaly, a condition in which, due to suppression of the development of the head, the head is reduced to a crumpled mass bent on the yolk (hence the name omphalocephaly) and the heart is situated anterior to the head. A typical case of omphalocephaly is illustrated in my Fig. 49. Dareste notes that omphalocephaly is nearly always accompanied by grave defects of the brain, eyes, and olfactory sacs. (8) Various modifications of the brain and neural tube. (9) Modifications of the eyes. (10) Celosomia or protrusion of the viscera ventrally, due to failure of the lateral limiting sulci to close below. (11) Failure of development of the limb buds and other abnormalities of the limbs.

From this review of literature it seems evident that the described abnormalities in the chick correspond with the expectations from the susceptibility data in-so-far as the latter are available. It has been shown in this paper that the parts of the embryo most susceptible to toxic agents and hence expected to be most amenable to modification are the anterior and posterior ends of the embryonic axis, the special sense organs, particularly the eyes, unions, crests of folds, bends, turns, etc., and the limb buds. It will be seen from the long list of abnormalities just reviewed that these parts are indeed those which most frequently exhibit abnormal development after subjection to a variety of experimental conditions. The susceptibility of certain other parts which seem frequently to exhibit abnormal

development, as for instance the area vasculosa, was not determined in my experiments and hence it cannot be stated definitely that the same explanation applies to such anomalies. However, it seems very probable that such is the case. The heart is treated in a separate paper which follows this one.

Finally it should be pointed out that both of two paired structures or the two halves of a median structure are not necessarily equally susceptible; consequently asymmetrical abnormalities are possible since, for instance, one eye may be slightly more susceptible than the other.

IV. Summary.

1. Differences in the time of death of different regions of chick embryos dying in toxic solutions are described. Chick embryos of various stages from the primitive streak through the third day of development were studied. In primitive streak and head process stages, there is a simple death gradient from the anterior to the posterior end of the embryonic axis. From the medullary plate stage on there are two regions of high susceptibility—the anterior end of the axis and the growing point of the primitive streak; from these two regions death progresses towards the middle of the embryo. Other structures showing high susceptibility at certain times are: the special sense organs, the limb buds, and all places where unions, folds, bendings, etc., are about to occur. A more detailed summary is given on p. 11.

2. Application of the results to normal development leads to the following suggestions, susceptibility or time of death being regarded as roughly proportional to metabolic rate:

a. There is a gradation in rate of metabolic activity along the primitive streak in an antero-posterior direction. This probably determines the cephalo-caudal progress of development.

b. The head process is probably merely a further development of the anterior end of the primitive streak.

c. The primitive streak (excluding the head process) forms all of the embryo from the first somite posteriorly.

d. Such developmental processes as the union of paired primordia, formation of bends and flexures, occurrence and

closure of folds, etc., result primarily from cell activity and not from mechanical causes.

e. Intense metabolism is a necessary condition for active development.

f. The chordate embryo in general develops through the formation of two centers of activity, one of which is situated at the anterior end of the embryonic axis and develops into the head, and the other of which, more posteriorly located, is a growing point which progressively shifts posteriorly, depositing the trunk of the embryo anterior to it. This posterior center of activity is the dorsal lip of the blastopore in amphibian embryos, that portion of the teleost embryo which is situated in the germ ring, and the differentiating region of the primitive streak in the chick.

g. Whether or not the chordate embryo develops with a primitive streak appears to depend on the relative time of appearance of the posterior center of activity with respect to the time of gastrulation. If the posterior center is established at the time of gastrulation, development occurs without the formation of a primitive streak; if later, a primitive streak arises.

3. A review of the literature on induced teratological development in the chick is given and an explanation of such development is suggested on the basis of the present results. It is pointed out that those portions of the embryo shown in this paper to be most susceptible to lethal solutions also most frequently exhibit abnormal development under the action of agents of a general depressing nature. Such agents or conditions in proper concentration and duration affect the course of development in rough proportion to metabolic rate, those parts of the embryo requiring the highest metabolic rate for their normal development or having the highest rate at the time of application of the condition being more affected than parts developing at lower metabolic levels.

BIBLIOGRAPHY.

Adelmann, H. B.

'22 The Significance of the Prechordal Plate: an Interpretative Study. Amer. Jour. Anat., Vol. 31, pp. 55–101.

Alsop, Florence M.

'19 The Effect of Abnormal Temperatures upon the Developing Nervous System in the Chick Embryo. Anat. Rec., Vol. 15, pp. 307–331.

Assheton, R.

'94*a* The Primitive Streak of the Rabbit, the Causes which may Determine its Shape, and the Part of the Embryo Formed by its Activity. Q. J. M. S., Vol. 37, pp. 191–222.

'94*b* On the Growth in Length of the Frog Embryo. Q. J. M. S., Vol. 37, pp. 223–243.

Bartelmez, G. W.

'12 The Bilaterality of the Pigeon's Egg. Part I. Jour. of Morph., Vol. 23, pp. 269–329.

Bellamy, A. W.

'19 Differential Susceptibility as a Basis for Modification and Control of Early Development in the Frog. BIOL. BULL., Vol. 37, pp. 312–361.

Byerly, T. C.

'26 Studies in Growth. I. Suffocation Effects in the Chick Embryo. Anat. Rec., Vol. 32, pp. 249–271.

Child, C. M.

'13 Studies on the Dynamics of Morphogenesis and Inheritance in Experimental Reproduction. V. The Relation between Depressing Agents and Rate of Metabolism in Planaria dorotocephala and its Value as a Method of Investigation. Jour. Exp. Zoöl., Vol. 14, pp. 153–206.

'25 The Physiological Significance of the Cephalo-caudal Differential in Vertebrate Development. Anat. Rec., Vol. 31, pp. 368–384.

Dareste, C.

'82 Sur la production des monstres dans l'oeuf de la poule par l'effet de l'incubation tardive. C. R. de l'Acad. Sci., Vol. 95, pp. 254–256.

'83*a* Nouvelles recherches sur la production des monstres dans l'oeuf de la poule, par l'effect de l'incubation tardive. C. R. de l'Acad. Sci., Vol. 96, pp. 444–446.

'83*b* Recherches sur la production des monstruosités par les secousses imprimées aux oeufs de poule. C. R. de l'Acad. Sci., Vol. 96, pp. 511–513.

'91 Recherches sur la production artificielle des monstruosités. Deuxième edition. Paris.

Edwards, C. L.

'02 The Physiological Zero and the Index of Development for the Egg of the Domestic Fowl, *Gallus domesticus*. Amer. Jour. of Physiol., Vol. 6, pp. 351–397.

Eycleshymer, A. C.

'98 The Location of the Basis of the Amphibian Embryo. Jour. of Morph., Vol. 14, pp. 467–480.

Faris, H. S.

'24 A Study of Pigment in Embryos of Amblystoma. Anat. Rec., Vol. 27, pp. 63–76.

Féré, C.

'94 Note sur l'influence de la temperature sur l'incubation de l'oeuf de poule. Jour. de l'Anat. et de la Physiol., Vol. 30, pp. 352–365.

'99*a* Le influence du repos sur les effects de l'exposition préalable aux vapeurs d'alcohol avent l'incubation de l'oeuf de poule. C. R. Soc. Biol., Vol. 51, pp. 255–258.

'99*b* Note sur l'influence de l'exposition préalable aux vapeurs d'ammoniaque sur l'incubation de l'oeuf de poule. C. R. Soc. Biol., Vol. 51, pp. 806–808.

Gerlach, L., and Koch, H.

'83 Ueber die Production von Zwergbildungen im Hühnerei auf experimentellem Wege. Biol. Centralbl., Vol. 2, pp. 681–686.

Gilman, P. K., and Baetjer, F. H.

'04 Some Effects of the Röntgen Rays on the Development of Embryos. Amer. Jour. Physiol., Vol. 10, pp. 222–224.

Hoadley, L.

'26*a* Developmental Potencies of Parts of the Early Blastoderms of the Chick. I. The First Appearance of the Eye. Jour. Exp. Zoöl., Vol. 43, pp. 151–178.

'26*b* Same. II. The Epidermis and Feather Primordia. Same, pp. 179–196.

'26*c* Same. III. The Nephros, with Special Reference to the Pro- and Mesonephric Portions. Same, pp. 197–224.

Hyman, L. H.

'16 An Analysis of the Process of Regeneration in Certain Microdrilous Oligochætes. Jour. Exp. Zoöl., Vol. 20, pp. 99–163.

'20 Physiological Studies on Planaria. IV. A Further Study of Oxygen Consumption during Starvation. Amer. Jour. Physiol., Vol. 53, pp. 399–420.

'21 The Metabolic Gradients of Vertebrate Embryos. I. Teleost Embryos. BIOL. BULL., Vol. 40, pp. 32–73.

'26 Same. II. The Brook Lamprey. Jour. Morph. and Physiol., Vol. 42, pp. 111–141.

Kaestner, S.

'95 Ueber die Unterbrechung der Bebrütung von Hühnereiern als Methode zur Erzeugung von Missbildungen. Verh. d. Anat. Gesellsch., 10th Versammlung, pp. 136–145.

Kingsbury, B. F.

'24 The Significance of the So-called Law of Cephalo-caudal Differential Growth. Anat. Rec., Vol. 27, pp. 305–321.

Lombardini.

'68 Forme organiche irregolari negli uccelli e ne batrachidi. Pisa.

Maggiorani.

'84 Atti R. Acc. dei Lincei. Travo 8. Abstract in Jour. Roy. Micro. Soc., Vol. 4, p. 861.

Mitrophanow, P.

'00 Teratogenese Studien. III. Einfluss der veränderten Respirationsbedingungen auf die erste Entstehung des Hühnerembryos. Archiv f. Entw'mech., Vol. 10, pp. 1–52.

Peebles, Florence.

'98 Some Experiments on the Primitive Streak of the Chick. Archiv. f. Entw'mech., Vol. 7, pp. 405–429.

Reese, R. M.

'12 The Effect of Narcotics upon the Development of the Hen's Egg. Sci., N.S., Vol. 35, no. 903, pp. 633–634.

Riddle, O.

'23 On the Cause of Twinning and Abnormal Development in Birds. Amer. Jour. Anat., Vol. 32, pp. 199–252.

Spemann, H., and Mangold, H.

'24 Ueber Induktion von Embryonalanlagen durch Implantation artfremder Organisatoren. Arch. f. mikro. Anat. und Entw'mech., Vol. 100, p. 599.

Stockard, C. R.

'14 The Artificial Production of Eye Abnormalities in the Chick Embryo. Anat. Rec., Vol. 8, pp. 33–38.

'21 Developmental Rate and Structural Expression. Amer. Jour. Anat., Vol. 28, pp. 115–278.

Tur, J.

'04 Sur les malformations embryonnaires obtenues par l'action du radium sur les oeufs de la poule. C. R. Soc. Biol., Vol. 57, pp. 236–238.

Wilson, J. W.

'25 The Disintegration of *Planaria maculata* in KCN. Abstract. Amer. Soc. Zoöl., Anat. Rec., Vol. 31, pp. 336–337.

Windle, B. C. A.

'91 Investigations in Artificial Teratogeny. Proc. Birmingham Philos. Soc., Vol. 7, part 2, pp. 220–232.

'93 On Certain Early Malformations of the Embryo. Jour. Anat. and Physiol., Vol. 27, pp. 436–453.

'95 On the Effects of Electricity and Magnetism on Development. Same, Vol. 29, pp. 346–51.

PLATE I.

FIGS. 1 TO 3. Three stages in the disintegration of a chick embryo in the primitive streak stage showing course of disintegration from anterior to posterior end of the streak.

FIGS. 4 TO 6. Chick embryo in the head process stage, Fig. 4, disintegration of the head process, Fig. 5, and course of disintegration posteriorly along the streak, Fig. 6.

FIGS. 7 TO 10. Medullary plate stage. Disintegration begins in the primitive knot, Fig. 7, and progresses posteriorly along the streak, Figs. 8 and 9. It then attacks the anterior end of the medullary plate, Fig. 9, and proceeds backward along the plate, Fig. 10.

FIGS. 11 TO 13. Early neural fold stage. Disintegration similar to the preceding.

FIGS. 14 TO 17. Stage of the first somite. Disintegration begins in the anterior end of the primitive streak, Fig. 15, and proceeds in both directions from this region. It then attacks the anterior end of the neural folds, Fig. 16 and progresses posteriorly meeting the other disintegration, Fig. 17.

FIGS. 18 TO 20. Three somite embryo. Disintegration begins in the differentiating anterior end of the primitive streak and in the middle of the neural folds. From both places it progresses forward and backward. The high susceptibility of the middle of the neural folds foreshadows the approaching fusion of these folds.

FIGS. 21 TO 25. Embryo of five somites, showing the double gradient, from the primitive streak forward and from the anterior end of the neural axis backward.

FIGS. 26 AND 27. Embryo of five somites in which the place of fusion of the neural folds is evidenced by heightened susceptibility.

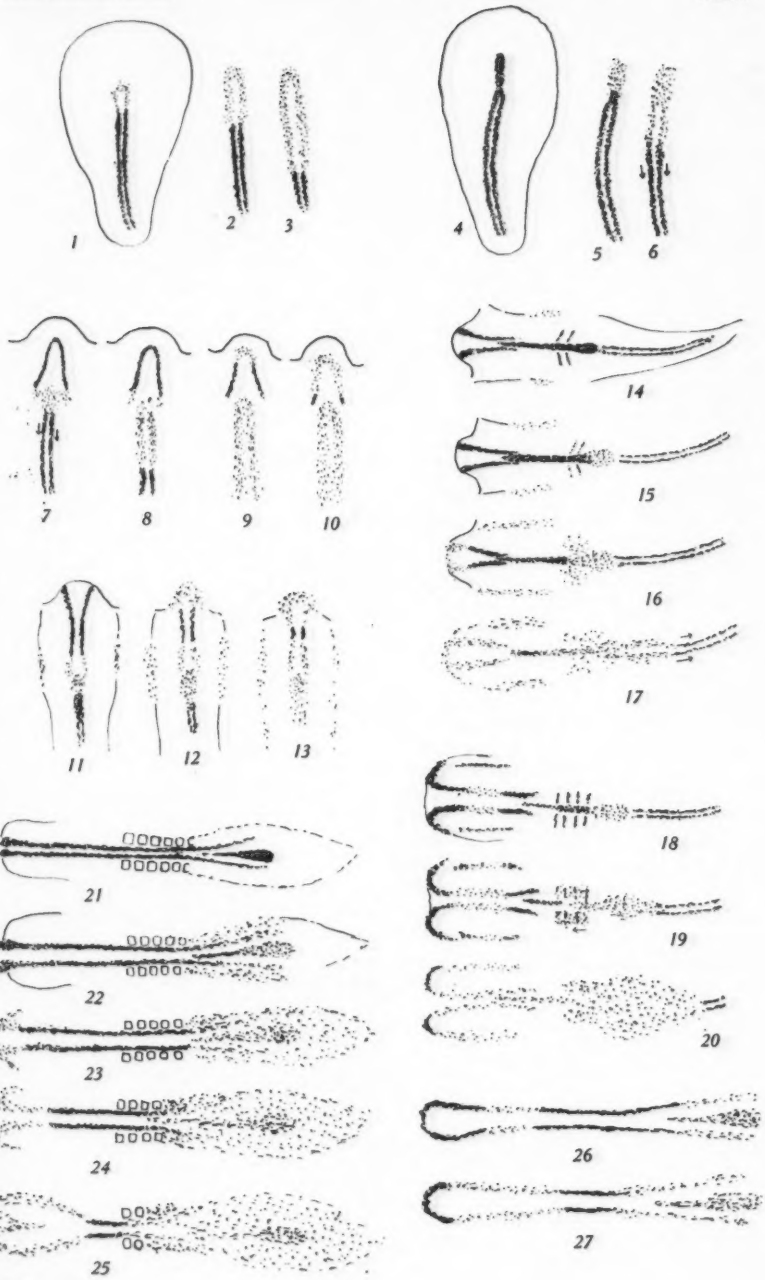

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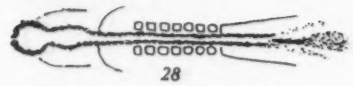
PLATE II.

FIGS. 28 TO 30. Embryo of seven somites, showing the usual double gradient of disintegration.

FIGS. 31 TO 35. Embryo of ten somites, showing the double gradient of disintegration and increasing susceptibility of the optic evaginations.

FIGS. 36 TO 39. Embryo of 12 to 13 somites showing general double gradient and area of increased susceptibility in the anterior end of the hindbrain foreshadowing the turning of the head.

FIGS. 40 TO 44. Fifteen somite embryo, with general double gradient; increased susceptibility of the optic stalks; increased susceptibility in the hind brain, more posteriorly located than in preceding figures, correlated with the turning of the head.



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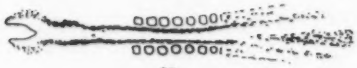

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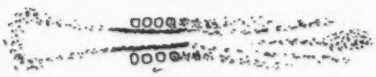

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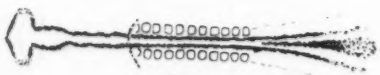

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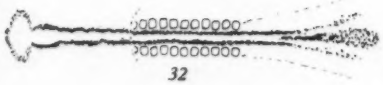

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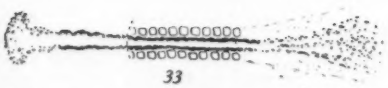

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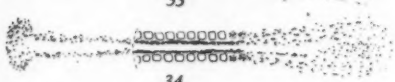

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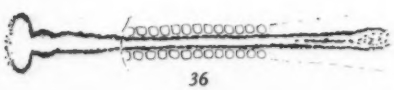

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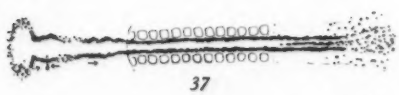

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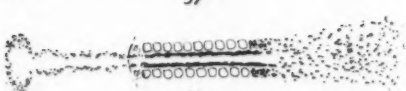

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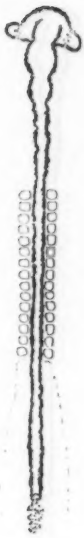

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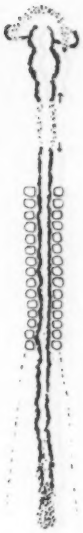

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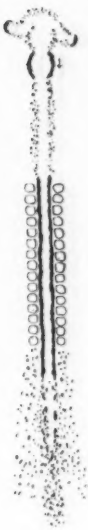

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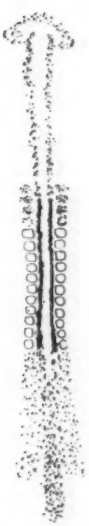

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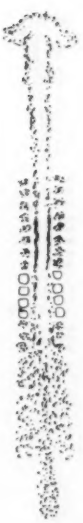

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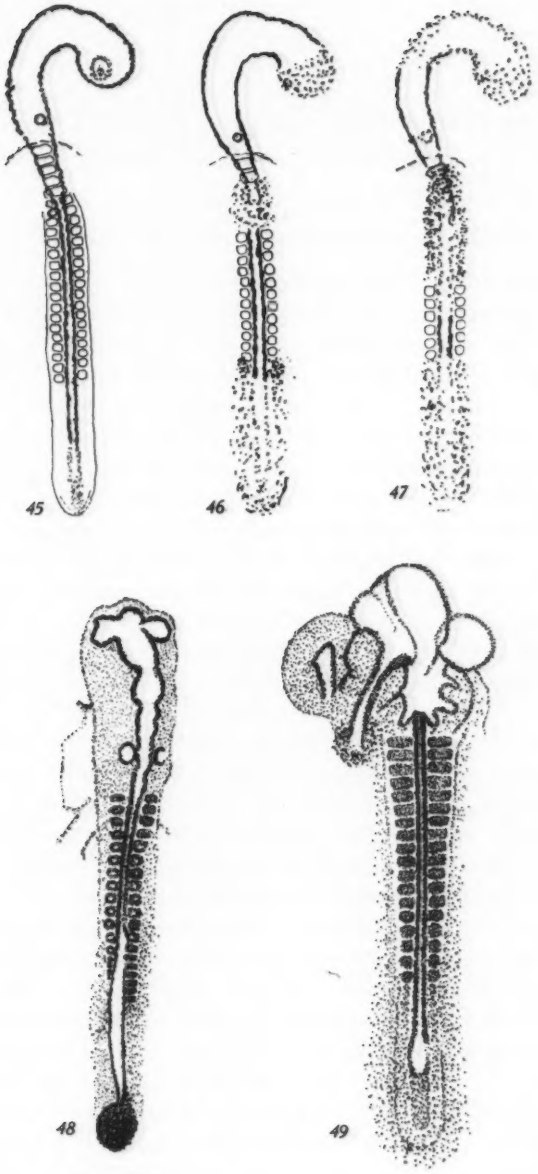
LIBBIE H. HYMAN.

PLATE III.

FIGS. 45 TO 49. Embryo of about two days incubation, with general double gradient, showing also high susceptibility of the eye and ear and of the region involved in the turning of the embryo.

FIG. 48. Abnormal chick resulting from retarded incubation. The anterior part is normal (except the heart), but the posterior end is retarded as shown by decreased number of somites on the left side, poor development of the neural folds, and large mass of cells terminating the embryo, due to inhibition of the growing region of the primitive streak.

FIG. 49. Typical case of "omphalocephalic" abnormality, due to delayed incubation. Posterior part nearly normal, but head reduced to a crumpled mass with heart anterior to the head.



LIBBIE H. HYMAN.

THE METABOLIC GRADIENTS OF VERTEBRATE EMBRYOS. IV. THE HEART.

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The study of the disintegration gradients of the heart was part of the work on the gradients of the chick embryo which has been presented in the paper which precedes this one in the same number of this journal. The investigation deals with the heart of the chick embryo only.

The methods employed were the same as recounted in the preceding paper of the series. The chick embryos were mounted ventral side up and the blastoderm which passes beneath the heart generally removed with a needle after the disintegrating agent had been applied and had begun to take effect. Unless this blastoderm is removed the heart cannot be seen very distinctly. The agents used were the same as in the preceding study, namely, potassium cyanide, ammonium hydroxide, and sodium hydroxide, all made up in rather strong concentration in isotonic sodium chloride solution.

It has proved necessary to spend a great deal of time and material in studying the death gradients of the chick heart. The reason for this is that the heart commonly dies without showing any very clear or obvious death changes. This seems to be due to some peculiarity of texture or consistency in the heart even in very early stages. Since the observer can naturally determine the time of death of a structure only when death changes of a visible nature occur, susceptibility conditions in the heart have been difficult to study. The clearest death changes occur in sodium hydroxide solutions but the same susceptibility differences have also been noted when ammonia or potassium cyanide solutions are employed.

Immediately after the establishment of the medullary plate the head of the chick embryo is delimitated from the blastoderm by the formation of the head fold. This fold composed of

entoderm and ectoderm is semicircular in outline, its concavity directed posteriorly. "The head fold thus produces an internal bay in the entoderm, the beginning of the fore-gut" (Lillie, "Development of the Chick," 2d edition, p. 91). The posteriorly advancing crest of the head fold thus marks the posterior limit of the fore-gut and constitutes the floor of the anterior intestinal portal.

When embryos of two or three to five or six somites are studied in disintegrating solutions, ventral side up, it is seen that the central part of the semicircular crest of the head fold is the most susceptible and that susceptibility diminishes from the center posteriorly along the margins of the head fold. This is shown in my Figs. 1 to 3.

At about the stage of four somites the mesoderm begins to invade the head fold as the so-called amnio-cardiac vesicles. These two cavities push in towards the median line. In the splanchnic wall of each amnio-cardiac vesicle where it lies adjacent to the entoderm of the advancing crest of the head fold, a mesodermal tube appears. These two tubes are as is well known the primordia of the heart. Upon studying embryos at this time, about five somites, the disintegration of this region is the same as earlier. The central part of the crest of the head fold is the most susceptible and the susceptibility diminishes along the posteriorly extending margins of the fold which now contain the heart tubes. The death of this region of a five to six somite embryo is shown in my Figs. 1 to 3. The metabolic conditions of the heart-forming region at this time are thus in harmony with the fact that the two heart primordia fuse from before backward, a high metabolic rate being essential for the process of fusion, as seen in the preceding paper.

By about the stage of seven somites the two heart primordia have fused, so that a small heart is present. But the crest of the head fold, now perhaps more correctly designated the floor of the anterior intestinal portal, still retains its high metabolic rate. Consequently after the heart tubes have united the point of high susceptibility and metabolism is still the middle of the crescentic fold which marks the posterior extent of the fore-gut. This region, however, *formerly* at the *anterior* end of the heart

tubes, is *now* at the *posterior* or *sinus* end of the heart. Consequently the death gradient in the heart now extends from the sinus towards the arterial end of the heart. In most of these early stages of the heart there is also still persistent more or less of a reverse gradient from the arterial end of the heart posteriorly. The extent of this differs in different hearts. In some it is very pronounced, in others scarcely evident. It seems probable that there is some growth in length at the arterial end of the heart.

The disintegration of a heart of a seven somite chick is shown in Figs. 4 to 8. Disintegration begins in the center of the posterior wall of the sinus, extends backward along the vitelline veins, forward along the heart towards the arterial end. At the arterial end there is a slight reverse gradient as shown in Figs. 6 to 8. Of four hearts of this age examined three were like the one illustrated while in the fourth the reverse gradient from the arterial end towards the sinus end was considerably more extensive.

At eight somite conditions in the heart are about as at seven somites, the reverse gradient being generally a little more pronounced. The typical course of disintegration at eight somites is illustrated in Figs. 9 to 11. Disintegration begins in the posterior wall of the sinus and progresses anteriorly along the heart. It then is initiated at the arterial end and proceeds towards the sinus end, meeting the other disintegration at about the middle of the heart. Of nine hearts of this stage examined, the reverse gradient was slight in three, extended about half way along the heart in four cases, and was entirely absent in one case. In one case there was hardly any difference in time of disintegration along the heart tube.

The heart of the nine somite stage is similar to the preceding but differs in one important particular. The disintegration commonly proceeds *faster along the right side* of the heart than the left. This is illustrated in the figures, numbers 12 to 16. Of nine hearts examined the right side was more susceptible than the left in five cases, in three cases the two sides were about equally susceptible, and in one case, the left side was more susceptible. In eight of them a reverse gradient from the arterial end towards the middle of the heart was present to a

greater or less extent. The greater susceptibility of the right than of the left side of the heart may already be evidenced at the eight somite stage. Its interpretation is considered immediately.

Two points may be emphasized here. First, in these early stages before the heart has begun to beat, there is already present a gradation in susceptibility, *i.e.*, in metabolic rate, from the sinus towards the arterial end of the heart. This gradient appears to originate from the backward growth of the anterior intestinal portal, which, at the time of the formation of the heart, takes place by the fusion from before backward of the splanchnopleuric margins of the head fold. Thus the direction in which the heart beat will pass along the heart seems to be determined by the manner of development of the fore-gut of the embryo. The second point concerns the greater susceptibility of the right side of the heart. This condition undoubtedly foreshadows the bending of the heart to the right which occurs subsequently. As emphasized in the preceding paper of this series all such bendings are preceded by a higher metabolic rate on the side which is to become the convex surface of the bend.

At the stage of ten somites the greater susceptibility of the right side is generally very pronounced. The disintegration of a heart of a ten somite chick is shown in Figs. 17 to 21. Disintegration proceeds from the sinus along the right side, later on the left side. There is generally still present a slight reverse gradient at the arterial end. Thirteen hearts of this stage were examined, of which eleven exhibited this reverse gradient, eight to the extent shown in the figures, three to a greater extent.

At about the stage of eleven somites the heart begins to bend to the right. This condition is marked by a high susceptibility of the middle of the right side. Disintegration begins as usual in the sinus end of the heart but soon attacks the middle of the right side; from this point it then extends anteriorly and posteriorly along the right side, and laterally towards the left side. The disintegration of a heart of eleven somites is shown in Figs. 22 to 26. From this time on the reverse gradient at the arterial end is either absent or very slight in extent.

Hearts of twelve to fifteen somites are generally similar as

regards their death gradients to the eleven somite heart, Figs. 22 to 26. In all a total of sixteen hearts of ages from eleven through fifteen somites were studied. In all of these the right side was more susceptible than the left, usually markedly so. In twelve cases, the gradient on the right side was of the type shown in Figs. 22 to 26, that is, disintegration extended from the middle of the right side anteriorly and posteriorly. In the other four the course of disintegration was similar to that shown in Figs. 17 to 21.

After the stage of fourteen somites the high susceptibility of the middle of the right side of the heart gradually lessens and has completely disappeared by sixteen somites, sometimes earlier. In hearts of embryos of sixteen to over twenty somites there is always present a simple gradient from the sinus to the arterial end of the heart. At this time of course the heart is beating regularly. It is interesting to note that the disintegration of the sinus always begins exactly at the places where the beat is initiated. The disintegration of a heart of a sixteen somite chick is shown in Figs. 27 to 31. The place of initiation of the beat is indicated in Fig. 27 by arrows. Ten hearts of chicks of sixteen to eighteen somites were observed and the course of disintegration was the same in all of them.

Conditions also continue the same from these stages through about the second day of development. Figs. 32 to 35 show the disintegration of a heart of a 20 somite chick. As before the sinus begins to disintegrate at the place where the beat originates and from there disintegration proceeds along the heart tube towards the arterial end where there is a slight reverse gradient. From the sinus death progresses caudally along the vitelline veins. Conditions remain the same up to about 25 somites, or the end of the second day of development.

From the second to the third day there is noticeable a greatly increased susceptibility in the ventricular region. The auricular evaginations also exhibit heightened susceptibility. Figs. 36 to 39 illustrate the disintegration of a heart of a thirty somite chick, about sixty hours of incubation. Disintegration begins at the tip of the ventricular bend and from there proceeds anteriorly along the bulbo-conus arteriosus and caudally towards the

auricular region. Meantime the auricular evaginations are disintegrating. The sinus region at this stage cannot be seen very well as it is buried in the body. Stages beyond the third day have not been studied.

It thus appears that there is in the heart before it has begun to beat a gradient in activity from the sinus towards the arterial end and that this gradient is the cause of the direction and sequence of the heart beat. This gradient appears to be established simply by the mode of formation of the heart by the backward growth of the anterior intestinal portal, just as a similar postero-anterior gradient is established at the rear end of the chick embryo by the backward growth of the growing point of the primitive streak, as set forth in the preceding paper. In the latter case, the gradient does not persist and hence this embryonic postero-anterior gradient in the axis leaves only morphological signs behind it, namely, the segmentation of the vertebrate body. There may be some functional paths of the postero-anterior type also in the nervous system as permanent records of the embryonic physiological history. But in the heart the postero-anterior, *i.e.*, the sin-arterial, gradient is permanently retained. Why? We cannot answer this question very well except to suggest that the isolation of the heart from the dominance of other structures permits it to retain its high embryonic rate of metabolism.

In the history of physiology, much effort, argument, and paper have been expended on the question of the "cause" of the heart beat, whether neurogenic or myogenic or due on further analysis to the chemical conditions within the heart. This question like many other biological questions has never received any adequate or satisfactory answer; and the reason for this is, as in many other cases, that the question is a false one, it is wrongly put. It seems to me to be really not of the slightest significance whether the heart beat be neurogenic or myogenic; the real question is: what property of any tissue makes that tissue automatic? The crux of the matter would seem to lie in the nature of stimulation. It is evident that some organs function without extrinsic stimulation, while others do not.

Elsewhere I have made a suggestion as to the essential nature

of the stimulated condition (Hyman, '18). I have pointed out that the chief difference between a stimulated and unstimulated organ is apparently one of rate of activity. A muscle or gland in a stimulated state does not appear to exhibit phenomena different from those characteristic of it in the unstimulated condition; it simply carries on its particular processes at a faster rate. If this be true then the essential feature of stimulation is an increase in the rate of processes in the organ stimulated. Granting this premise it follows that any organ whose rate of activity is already sufficiently high will of necessity be automatic. From this point of view the cause of the heart beat may be expressed simply as follows: certain or all of the heart tissue has so high a rate of activity that it functions in the absence of extrinsic stimulation. Now it is not of any consequence whether the tissue of the heart which possesses this high rate of activity is muscle or nerve. The state of affairs in this regard differs in different hearts. It is highly probable that in all hearts when they begin to beat in the embryo the automaticity resides in the muscle cells. But the muscle cells may lose their high metabolic rate with age and in that event the aid of the nervous system must be partially or wholly evoked to keep the apparatus going. The nervous system appears to be characterized by a high metabolic rate and is thus able to control absolutely or alter the rate of activities of other organs. In the process of ontogeny it comes to exercise more or less complete control over the heart, the degree varying in different animals.

It remains to consider briefly the teratology of the heart. Dareste in his book ('91) discusses only one type of anomolous heart in the chick, namely, the duplicature of this organ. This condition as he correctly points out results from a failure of the two heart primordia to unite. This union begins at the anterior end of the two heart tubes, a region of high susceptibility as shown in this paper. Depressing conditions would undoubtedly inhibit this union in whole or part resulting in the occurrence of partially or completely doubled hearts.

On the basis of the susceptibility data presented in this paper one might also expect certain other abnormalities of the heart. Thus I have shown that at an early stage of the heart the right

side of the heart is more susceptible than the left. This greater activity of the right side results in the bending of the heart to the right. Depressing conditions would tend to prevent this bending, so that the heart would remain as a straight tube. I have also shown that in general the sinus end of the heart has the higher rate of activity and that this decreases towards the arterial end. Inhibiting factors might be expected to affect the sinus end of the heart more than the arterial end, so that hearts with small sinuses and enlarged arterial ends might be produced.

Both of these expectations have been realized in certain abnormal chicks produced during my study. These chicks have already been referred to in the preceding paper. They arose as a result of incubation for several days at 30° C., followed by incubation at 39° C. for 24 hours. That development had occurred at 30° is proved by the fact that all of these chicks showed a degree of development corresponding to 40 to 72 hours of incubation. All of this lot of chicks were abnormal and in many of them the heart was abnormal. As the other abnormalities were considered in the preceding paper, only the heart concerns us here. The abnormalities were of two kinds: (1) the persistence of the straight form of heart tube at a stage of development in which the heart would normally be considerably curved; and (2) a reduced sinus region with an abnormally enlarged arterial end. The first type of abnormality is illustrated in my Fig. 40. This embryo has thirteen somites but the heart lacks the normal curvature. The arterial end is also abnormally large. Fig. 41 is typical of the second kind of abnormality. In this embryo of seventeen somites the sinauricular region is quite small as compared with the normal but the anterior part of the heart is considerably enlarged. This heart also does not bend to the right to the normal extent. It should be stated that all of these hearts were living and beating at the time when the embryos were fixed.

It thus appears that the teratological development of the heart corresponds with the expectations on the basis of the susceptibility results.

SUMMARY.

1. Before the formation of the heart the middle of the crest of the head fold is highly susceptible and the susceptibility decreases along the posteriorly directed margins of this fold which contain the primordia of the heart. This finding is in harmony with the fact that the two heart primordia fuse from in front backward.

2. After the heart is formed the anterior intestinal portal, now situated at the posterior end of the heart, retains its high susceptibility, and this fact appears to induce a corresponding direction of the gradient in the heart. In the heart at all stages from its first formation (about seven somites) to the latest stage studied (three days) there is a general susceptibility gradient from the sinus towards the arterial end. This gradient appears to originate from the manner of formation and backward progression of the anterior intestinal portal. In early stages of the heart there is usually also present a reverse gradient at the arterial end, due probably to the persistence of conditions stated in 1.

3. There is thus in the heart before it has begun to beat a sin-arterial gradient in metabolic rate. This is probably the cause of the sequence of the heart beat.

4. In addition to the general sin-arterial gradient, secondary regions of heightened susceptibility appear at times. The most important of these is the increased susceptibility of the right side. This begins at about the nine somite stage and persists until about fifteen somites. It is correlated with the curvature of the heart to the right. Between the second and third day ventricles and auricular evaginations show temporary local increased susceptibility.

5. Described abnormalities of the heart can be correlated with these findings on differential susceptibility along the heart.

BIBLIOGRAPHY.

Dareste, C.

'91 Recherches sur la production artificielle des monstruosités. Deuxième edition. Paris.

Hyman, L. H.

'18 Suggestions Regarding the Causes of Bioelectric Phenomena. Sci., N.S., Vol. 48, No. 1247, pp. 518–24.

PLATE I.

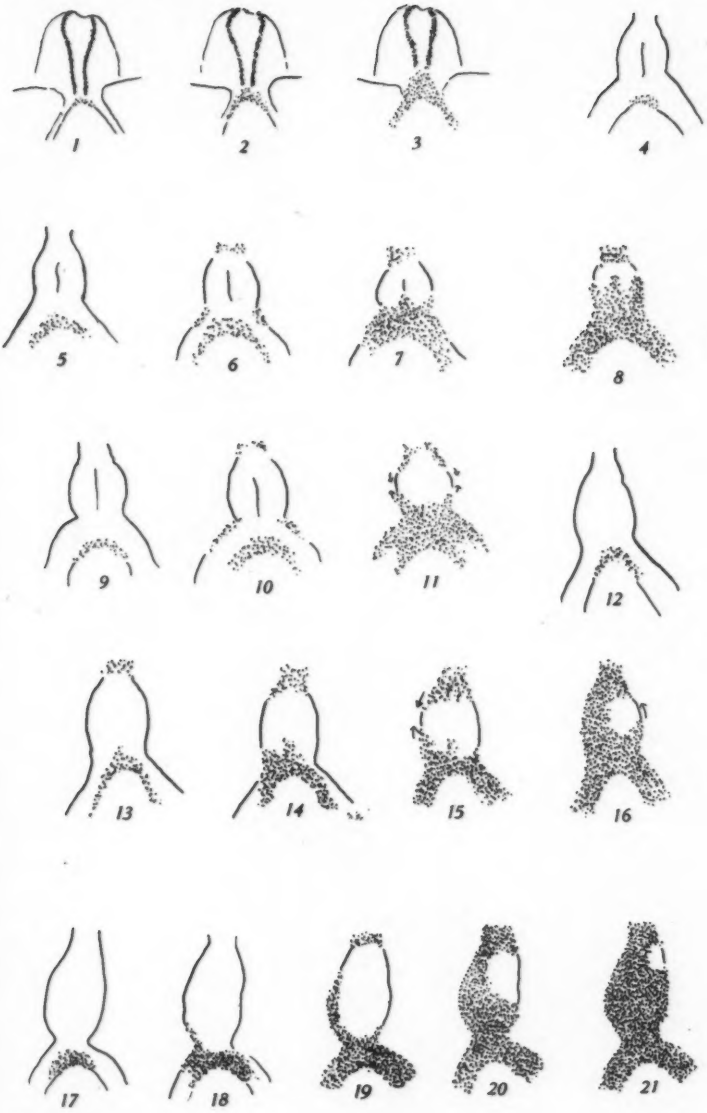
FIGS. 1 TO 3. Ventral view of anterior end of an embryo of five to six somites, showing the disintegration of the crest of the head fold.

FIGS. 4 TO 8. Five stages in the disintegration of a heart of a seven somite chick, showing the sin-arterial gradient with a slight reversal at the arterial end.

FIGS. 9 TO 11. Course of disiategration of an eight somite heart, similar to the preceding, but with a more extensive reversal at the arterial end.

FIGS. 12 TO 16. Disintegration of a nine somite heart, showing general sin-arterial gradient and greater susceptibility of the right side as compared with the left.

FIGS. 17 TO 21. Disintegration of a ten somite heart, similar to the preceding, the greater susceptibility of the right side very marked.



LIBBIE H. HYMAN.

PLATE II.

FIGS. 22 TO 26. Disintegration of a heart of eleven to twelve somites showing increased susceptibility of the middle of the right side.

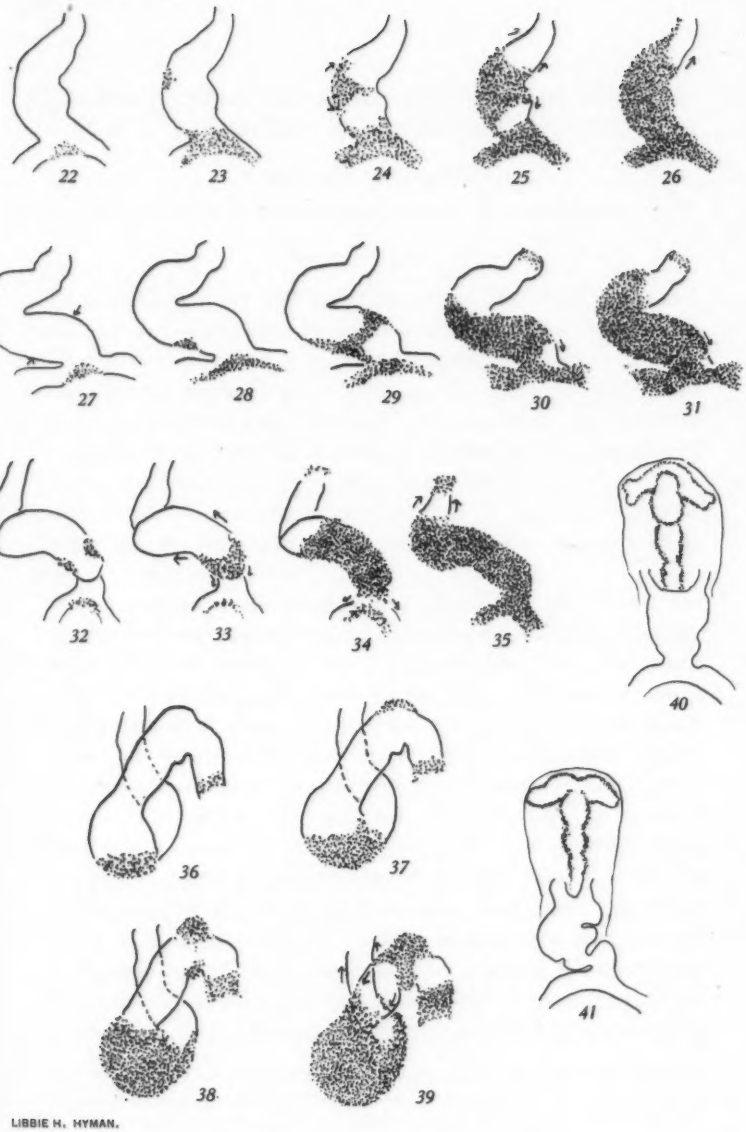
FIGS. 27 TO 31. Heart of a sixteen somite chick, showing restoration of simple direct gradient from the sinus to the arterial end. Disintegration in the sinus begins at the exact places of initiation of the heart beat, indicated in Fig. 27 by arrows.

FIGS. 32 TO 35. Heart of twenty somites, similar to the preceding.

FIGS. 36 TO 39. Heart of thirty somites, showing heightened susceptibility of the ventricular bend and the auricular evaginations, indicating rapid development of these regions at this time.

FIG. 40. Ventral view of an abnormal heart of a chick embryo of thirteen somites, obtained by incubation at subnormal temperature, showing inhibition of the bend to the right, and abnormally enlarged arterial end. The nervous system is normal but the posterior end of this chick (not shown in the drawing) is inhibited as discussed in the preceding paper.

FIG. 41. Ventral view of another abnormal chick of the same lot as Fig. 40 showing reduced sinus region as compared with arterial region. This chick also has an inhibited posterior end, not shown.



LIBBIE H. HYMAN.

EXPERIMENTS IN REARING COLONIES OF BUMBLE-BEES (BREMIDÆ) IN ARTIFICIAL NESTS.[1]

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INTRODUCTION.

In a detailed study of the life history of the bumblebees and their inquilines and parasites, it is obviously impossible to secure all the desired information from field observations alone. Therefore, various methods of procedure are necessary and some laboratory methods must be used. An account of the methods developed for the rearing of colonies of these social bees during five years of study may seem superfluous, but in view of previous publications, the ever-increasing interest in these insects, and their economic importance, such an account has its practical as well as academic value.

Until comparatively recent times practically all of our biological knowledge concerning the activities associated with nidification were the result of the chance discovery of nests, their hasty examination, and more rarely their observation for a limited period of time. To a large extent the classical observations of Réaumur (1742), Huber (1801), Lepeletier (1836), Putnam (1864) and many others were the result of this type of study. It is but natural, then, that the information so accumulated should relate for the most part to the size of the colonies, the inquilines and parasites found in the nest when opened, the arrangement of the comb, and various other readily apparent details of the nest economy. Such a method, however, can not be depended upon when a more detailed study of the life history of the individual bees and the development of the colony of one or more species is desired. Furthermore, the fact must not be

[1] Contribution from the Entomological Laboratories of the University of Illinois, No. 106. Extract number three from a thesis presented to the Faculty of the Graduate School of the University of Illinois in May, 1923, in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

overlooked that the vast majority of the colonies discovered in their natural environments are found at a time of the year when little can be learned about many of the aspects of their life history; the starting of the colony, for example.

Hoffer (1882–1883) says on page eleven of the first part of his celebrated publication that he has never seen how the comb is started and adds that apparently neither has any other observer. In the appendix of the second part of this work, however, Hoffer tells how to his great joy he finally observed the start of the comb of *B. lapidarius*. In this latter instance the queen was under confinement and the information obtained by Hoffer concerning this phase of the development of the comb was not based on field observations. Westerlund (1898) has given us a short account of a nest in its incipient stages that he found in Finland and v. Buttel-Reepen (1903) gives a few notes on the subject furnished him by Herr Wegener. Since then Wagner (1907), Lindhard (1912), Sladen (1912), Armbruster (1914) and Plath (1923) have added to our knowledge concerning the start of the comb. Sladen and Plath give the best accounts of the start of the nest and comb thus far published. The correctness and completeness of the descriptions of these last mentioned writers are due to the fact that they did not rely upon field observations alone.

By calling attention to the disadvantages incurred by depending upon field nests and the limitations of such studies I do not mean to imply that the examination of such nests is not or has not been of importance. I merely wish to point out that by so doing one is relying to a great extent upon chance and therefore a means which does not guarantee results to one intent upon penetrating deeper into many of the details, characteristics, and mysteries of the life history of the bumblebees.

In a comparatively recent number of the BIOLOGICAL BULLETIN (1923), Mr. O. E. Plath has published an exceedingly interesting and valuable account of his "Breeding Experiments with confined *Bremus* (*Bombus*) Queens." In the introductory part of this paper Plath adequately reviews the experiments of Hoffer (1882), Lindhard (1912), Sladen (1912), and Frison (1918) of this nature, and accordingly these papers need not be reviewed again.

CONSTRUCTION OF ARTIFICIAL NESTS AND TECHNIQUE.

My first serious experiments in confining the queens began in the spring of 1915 and were continued each spring of 1916, 1917, 1919, and 1920. As early as 1910 I tried to get queens to start nests in confinement, but the methods then employed were so poor that I will not review these futile attempts. It was not until 1917 that I succeeded in getting colonies by this method (1917). For confining the queens I used, in 1917, a small wooden box 5 inches by 7 inches by 2 inches. At one end of this box was a round opening which could be closed by the insertion of a cork. For the top I used small ruby or amber colored glass sections. During the intervals between examinations a dark cloth was wrapped about the box to exclude the light. In one corner of the box remote from the grass nest was placed a small tin, in which liquid food was supplied to the queen. In 1919 and 1920, I used boxes for confining the queens similar to those used in 1917.

Within these boxes I used various materials for making the conditions such that a queen would be induced to start a colony. Naturally, because the queens of bumblebees select the nests of field mice the first material used was the soft, day grass from such nests. Field mice always select a fine quality of grass for their nests and hence grass from such a source needs no further sorting or treatment. The nests, however, are not always easy to find and oftentimes I was hard pressed to get enough of the grass from this source for my experiments. Sladen experienced the same difficulty in getting suitable grass to use in domiciles for attracting bumblebee queens. I found that the best time and place to look for nests of this type was in early spring along a railroad right of way, after the ground had been burned over to destroy the coarser vegetation. The nests so located usually escaped destruction by the fire and were then easily perceived. The difficulty experienced at times in getting enough of these nests, led me to try to find a substitute for the finely sorted grass they contained. Cotton did not have enough "body" and the small cotton fibers became enmeshed continually with the pollen supplied the queen. The silk from milkweed pods likewise proved to be too light and fluffy, as was the case also with raw

wool. In several instances I used in my boxes without alteration, except for the addition of a top covering, the nests of small birds such as those made by field sparrows.

Bumblebees always keep the brood and comb well covered, in order to exclude light, avoid temperature fluctuations, etc. When making daily observations on a colony I always found it necessary to remove the covering of the nest. When it was replaced again the workers or queen would immediately start to work and repair it. To save the bumblebees the daily repetition of repairing the top covering, I devised a square pad of four or five layers of cheese cloth, fastened these together and then placed this pad over the comb. This gave very good results. All I needed to do in order to see the contents of the nest was to lift the cheese-cloth pad, and then upon finishing my observations, replace the same. The bumblebees readily accepted this substitute for the covering of their own making and in so doing saved themselves from constantly repairing the top. As was the case with the grass top, the bumblebees plastered a thin coat or layer of wax and pollen composition on the side of the pad adjacent to the brood chamber. Later I went a step farther and coated one side of the cheese cloth pad with pure melted honeybee wax before placing it over the nest. This seemed to break the bumblebees of their habit or instinct of coating the pad with wax and pollen and hence resulted in a considerable saving to them of time and materials. The fact that the bumblebees accepted the artificial wax-lined cheese cloth pad without material change is a good indication that it fulfilled all the requirements of their instincts in this direction.

The artificial top proved to be such a decided success that I next tried to make the whole nest of cheese cloth coated on the inside with wax. I first tried lining the bottom of the box with cheese cloth, and then lining this with the melted wax. Then I placed over this a dome-shaped cover of wax-coated cheese cloth. Of course an entrance into this enclosed chamber was provided. Because of certain undesirable features I abandoned this method. Finally, I resorted to lining merely the bottom and sides of the back part of the box with four or five layers of cheese cloth and then coating this with melted wax. For the

back and ceiling a single elongate pad of the right dimensions was made in the same manner as before, and then fastened to the back of the box so as to form a stationary back part and a movable covering. This could then be lifted up from the front in much the same way as one would lift the hinged lid of a box. This top pad was easily pinned to the floor in the fore part of

FIG. 1. Start of the comb of *Bremus impatiens* under controlled conditions, showing: *a*, the pollen lump containing the first egg cells and eggs; *b*, the artificial honey pot. May 2.

the box, thus enclosing a neat light-proof, wax-lined and soft-walled chamber. An entrance to this enclosure was provided by cutting out a part of the top covering, or merely by pinning to the side of the box one corner of the free end of the top.

The adoption by the bumblebee queens of this last-described artificial nest relieved me of many of my earlier worries. I no longer needed to search about in spring for the nests of field mice, for this artificial nest seemed to fulfill in every way all the

requirements of the nesting instincts which are so pronounced in bumblebee queens looking for a place to adopt as their home. Further, I could purchase very cheaply all the material that I needed for making these artificial nests. As a saver of time the artificial nest was a great improvement over anything I had ever used before. The nests could be made up at any time of the year, in any number and during odd moments. Formerly, I had to look for grass suitable for my purpose in spring, the very busiest time of the year for one attempting a biological study of bumblebees.

A slight variation of this artificial nest was often used. It consisted essentially of dividing the box into two sections, by means of a wooden partition. One of these sections was then made into an artificial nest as described above and a hole made in the partition so that the bumblebees could go from one section to the other. The remaining section was then used solely as a feeding chamber.

The liquid food which must be supplied to a queen bumblebee in confinement was kept in a small tin container. In the unpartitioned box this container was placed in one corner, and in the divided type in the section adjacent to the artificial nest. The liquid food supplied to the confined queens was simply pure, strained honeybee honey diluted with water. A mixture of sugar and water is eagerly consumed by hungry queens, but does not compare for this purpose with diluted honey, principally because it is not so concentrated. Adult bumblebees as well as the larvæ feed on pollen, but sometimes in confinement the queens can not be induced to eat the honeybee pollen. To defeat this indisposition on the part of the queens to feed on pollen, I occasionally added a small amount of rye flour to the diluted honey. In this way I hoped the queens would get some of the rye flour when lapping up the honey mixture. Pollen is rich in nitrogen and when eaten by the queens in spring probably contributes to the development of the eggs, causing the queen to become "broody" and finally to seek a home. Rye flour is also rich in nitrogen, and hence might aid in bringing about the same effect. The receptacles for the liquid food should permit as little exposure of the liquid as possible. Bumblebee queens

frequently climb up the sides of the box and then, reaching the top and unable to go farther, fall down again. If by chance they get sticky because of falling into the tin cover containing the honey, there is less chance of the queen starting a colony. Bumblebee queens under natural conditions keep themselves scrupulously clean. Any substance such as honey which mats

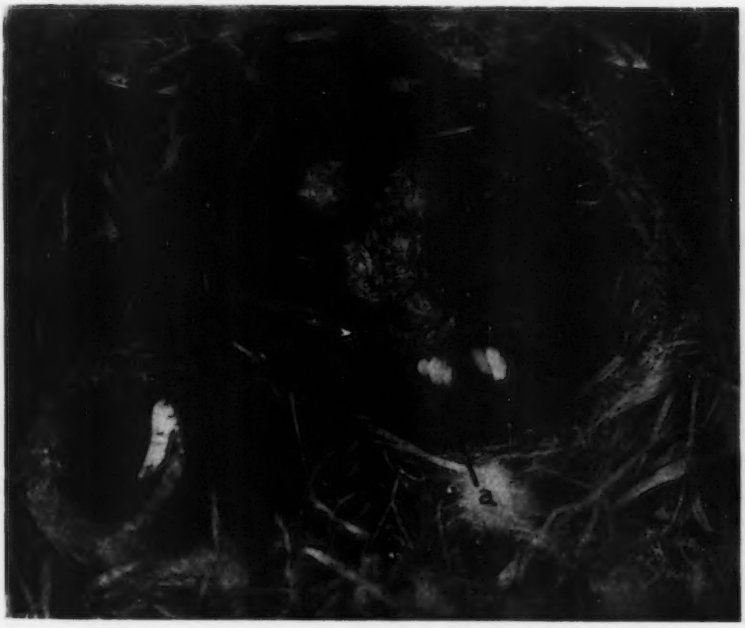

FIG. 2. Comb of *Bremus impatiens* of about two weeks development, showing: *a*, several eggs in the same cell; *b*, one of the first larval cells; *c*, groove caused by the queen brooding on the first larval cells. May 10.

down their glossy pubescence and becomes a "catch-all" for particles of dirt readily tends to make the queens dissatisfied with their quarters and as a result no comb is started. I often partially covered with stiff paper the tin lid containing the honey, leaving but a little space for the insertion of the proboscis of a bee. Almost any contrivance of this nature would suffice. If one were to confine large numbers of queens it might be profitable to devise a neater and more efficient type of feeder.

Not too much of the diluted honey should be made up at one time as it rapidly sours. I found it convenient to make up this mixture by filling a four-ounce bottle about three fifths full of honey, adding enough distilled water to nearly fill the bottle and shaking it until the honey was thoroughly diluted. A cork stopper provided with a medicine dropper was useful for drawing the diluted honey from the bottle and refilling the tin containers in the nesting boxes.

Besides liquid food, pollen is essential to a brooding queen for her personal consumption and for the proper development of her off-spring. Honeybee pollen is apparently as wholesome for bumblebees as for honeybees. One spring I tried to secure fresh pollen by removing the pollen from the corbiculæ of worker honeybees which I caught in the field. This required too much time for the quantity secured; in fact, did not produce enough for my needs. Therefore, I relied mainly upon getting the pollen from the brood frames of the honeybee. The pollen should be carefully removed, so as to avoid being mixed with foreign substances, and kept in bottles until needed. If it dries out it can be moistened again with honey. I am much indebted to Dadant and Sons, of Hamilton, Illinois, for gratuitously furnishing me with pollen in the spring of 1920.

There is no actual difference between the pollen stored by the honeybee and that of the bumblebee unless it be in the nature of the substance or fluid used to moisten the pollen in order to make it adhere to the pollen plate. This is evident from the fact that much of the pollen gathered by both bumblebees and honeybees is from the same flowers.

Before using the pollen I mixed it with enough honey to give it the consistency and plasticity of pollen freshly scraped from the legs of a foraging bee. I then placed a lump of the pollen about the size of a cherry seed in the artificial nest. This corresponded to the pollen mass collected by a queen under natural conditions when about to lay her first eggs. Besides this, I usually placed a small amount of the pollen near the liquid food as an extra source of pollen supply. That placed in the nest was intended primarily for the queen to use in building her first egg cells, but the use of this also as food did not complicate matters.

Another feature of the artificial nest was the honey pot. This was made from pure melted beeswax and modeled to imitate in every respect the honey pot first constructed by a nesting queen. I fastened this to the bottom of the nest by means of melted wax, just about one half inch distant from the pollen lump which was to serve as a future egg receptacle. Diluted honey was used for filling the honey pot. Naturally,

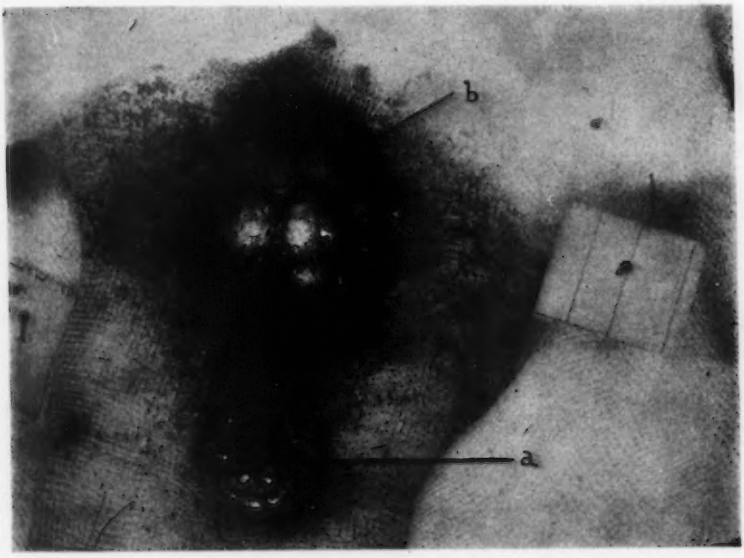

FIG. 3. Start of the comb of *Bremus vagans* in artificial nest, showing: *a*, artificial honey pot; *b*, first larval cells. June 21.

all the wax used by wild bees is produced by them, but to make conditions even more favorable I placed a few loose bits of pure beeswax near the artificial honey pot for the use of the queen if she so desired.

Considering the artificial nest as described above with all its parts, we find that it provides a queen with everything necessary for starting and eventually developing a colony. The wax-lined, cheese cloth chamber in the small wooden box in a general way corresponds to and possesses the same advantages as the

nest of a field mouse. Under natural conditions after finding a suitable nesting situation the queens must gather pollen and nectar. The honey pot is constructed of pollen and wax and filled with honey, shortly after the pollen is gathered for the nucleus of the first brood mass. Occasionally I moistened a

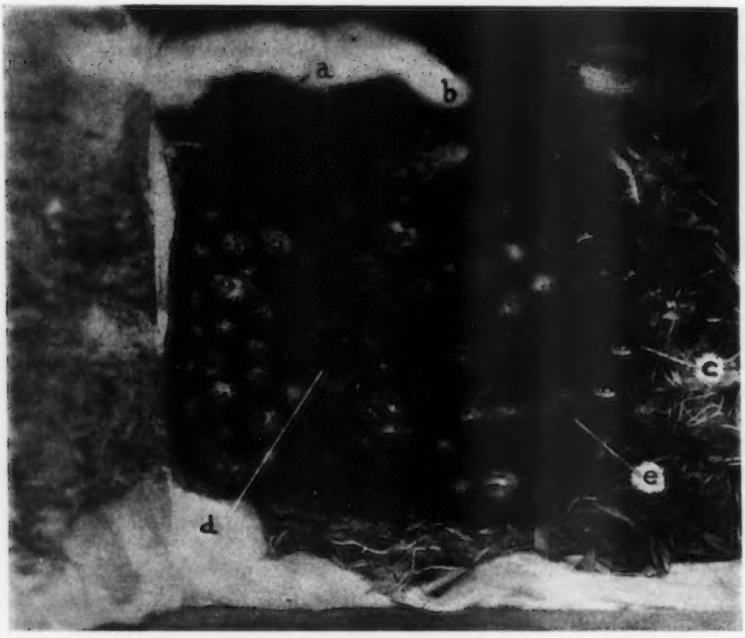

FIG. 4. Comb of *Bremus vagans*, showing: *a*, larval cells; *b*, old queen; *c*, wax-pollen pots used for the storage of honey; *d*, cocoon used for the storage of pollen; *e*, larval cell showing opening through which food is injected into the cell. September 16.

portion of the nest-lining with honey just to give it a bee-like odor. After I had placed an artificial honey pot, little bits of wax, and a small, compact mass of pollen in my artificial nest, there remained for the queen bent on laying eggs only the construction of the egg cells. Bumblebee queens under natural conditions avoid as much labor as possible. This is shown by the selection of nests of field mice, birds and other already

prepared quarters. Therefore, the elimination of the work of selecting a nest, the construction of a honey pot, and the collection of nectar and pollen should prove no obstacle to inducing the queens to start colonies in confinement. In fact, my experiments prove that such provisions are the secret of success in rearing bumblebee colonies in captivity. The method involved is simply a case of substituting artificial materials and objects for those naturally found or made by the queens. In my artificial nest as described above the queen finds herself in a position or environment which would be attained naturally only after days of patient labor. The instincts associated with the early start of a colony are so fixed that the queens often proceed with work in these artificial nests that is unnecessary. As an example of this I may state that bumblebee queens under such conditions often proceed to make changes in the artificial honey pot and in some instances build a new one.

The artificial nests should be prepared in sufficient numbers before the queens are on the wing in spring, except that the pollen lump should not be placed in the nest until the queens are introduced. I have always kept a close watch on the advance of the spring season and almost the first day of the appearance of the queens caught them for my experiments. In the vicinity of Urbana, Illinois, the queens of various species appear at different times and in order to get queens of all the species commonly found in this locality, it is necessary to prolong the collecting period over an extended time. The queens were captured in an ordinary insect net and then brought alive to the laboratory in mailing tubes. On arriving at the laboratory the queens were immediately removed and placed in large glass aquarium jars. Corrugated paper was placed on the bottom of these jars to afford the queens a suitable walking surface and the top of the jar was covered with several layers of cheese cloth to prevent their escape. Food was supplied them by scattering small bits of pollen about the bottom of their prison and by placing diluted honey in a tin-container fastened in the center of the corrugated bottom. In these jars the bees found plenty of food and ample opportunity for exercise. Besides keeping the queens at all times well fed, exercise seems to be essential.

Therefore, the larger the aquarium jars the better. If the queens are kept in too close quarters, even though provided with sufficient food, they soon become lethargic and die. Queens kept in the right type of aquarium jars and well fed soon become "broody." By "broody" I mean that the physiological processes going on within the bodies of the queens cause them to display characteristic nesting instincts. This is externally expressed by the production of wax, embracement of lumps of pollen in the jar, and excited buzzing and agitated movements when disturbed. Some species respond more quickly to this treatment than do others.

As soon as the queens became broody I put them in artificial nests. Usually two queens were placed in each nest, but I conducted also ten experiments in which only one queen was placed in each nest. Needless to say, when two queens were placed in the same nest, they were usually of the same species, but in some cases I purposely placed queens of different species in the same nest-box. Before introducing two queens into the same nest it is advisable to keep them in the same aquarium jar. In this way they appear to acquire the same characteristic odor and when placed in the nest the danger of the queens fighting is somewhat lessened. Where two queens were kept in the same box, in order to be able to distinguish them apart, I marked one of them by clipping a notch in her wings. In this way it was possible to keep an accurate account of the actions of each queen. Each artificial nest was given a number when one or two queens were introduced into it and observations made and notes taken as often thereafter as possible.

Results.

In Table I. is given the results that I obtained in getting queens to start colonies in captivity. In 1915 and 1916, the methods employed were very unsatisfactory and the fact that I did not succeed in starting a single colony is not surprising. Under "Remarks" in Table I. it is to be observed that in 1915 in two of the experiments the queens at one time gave some evidence of being interested in the nest. Though these experiments are classed as failures they, nevertheless, gave me many suggestions

TABLE I.

Species.	Experiment Number.	Number of Queens and Workers.	Date Experiment Started.	Results.	Remarks.
B. impatiens.....	1	1 queen	April 30, 1915	Queen died May 25, 1915	Manifested slight interest in the nest
B. auricomus.....	2	1 queen	April 30, 1915	Queen died June 23, 1915	No interest shown in the nest
B. americanorum....	3	1 queen	April 30, 1915	Queen dead May 5, 1915	No interest shown in the nest
B. impatiens.....	4	1 queen 3 workers	May 25, 1915	Queen dead June 1, 1915	Interest taken in the nest at first, especially by workers
B. americanorum....	5	1 queen	April 23, 1915	Queen dead April 30, 1915	No interest shown in the nest
B. impatiens.....	6	1 queen	April 23, 1915	Queen dead May 2, 1915	No interest shown in the nest
B. impatiens.....	7	1 queen	May 5, 1916	Queen dead May 7, 1916	No interest shown in the nest
B. separatus.....	8	1 queen	May 5, 1916	Queen dead May 27, 1916	No interest shown in the nest
B. bimaculatus.....	9	2 queens	April 17, 1917	Colony successfully started and reared	
B. bimaculatus.....	10	2 queens	April 17, 1917	One queen died May 1, 1917 One queen dead May 10, 1917	Interest manifested in the nest, but no eggs or cells. First queen killed by the other queen
B. bimaculatus.....	11	2 queens	May 5, 1917	One queen died May 11, 1917	No interest shown in the nest
B. bimaculatus.....	12	2 queens	May 13, 1917	One queen stung and partially paralyzed on May 16, 1917. Other queen released later	One queen took an interest in the nest about the time the first queen was stung
B. bimaculatus.....	13	1 queen	April 19, 1917	Placed with Exp. 14 on April 27, 1917	Interest taken in the nest as shown by making a "cozy" pocket in the nest, but then abandoned
B. bimaculatus.....	14	1 queen 1 queen	April 19, 1917 April 27, 1917	Colony successfully started and reared	
B. bimaculatus.....	15	2 queens	April 20, 1917	One queen died May 9, 1917 One queen released on May 15, 1917	No interest shown in the nest
B. bimaculatus.....	16	2 queens	April 20, 1917	One queen stung and killed on April 23, 1917. One queen released on May 5, 1917	One queen interested in nest on April 23, 1917, but lost interest after that date
B. separatus.....	17	1 queen 1 queen	April 20, 1917 April 24, 1917	One queen dead on May 13, 1917. Other queen placed in Exp. 18 on May 18, 1917	No interest shown in nest

TABLE I.—*Continued.*

Species.	Experiment Number.	Number of Queens and Workers.	Date Experiment Started.	Results.	Remarks.
B. separatus.....	18	2 queens	May 18, 1917	One queen stung and paralyzed on one side on May 24, 1917. One queen released on June 26, 1917	No interest shown in nest
B. americanorum....	19	2 queens	May 15, 1917	One queen dead on May 23, 1917. One queen released on June 3, 1917	No interest shown in nest
B. americanorum....	20	2 queens	June 21, 1917	One queen dead on July 5, 1917. One queen released on July 8, 1917	No interest shown in nest
B. bimaculatus.....	21	2 queens	April 17, 1917	One queen killed on June 11, 1917 after several workers had emerged. Colony successfully reared	
B. auricomus.....	22	2 queens	May 14, 1917	One queen removed after first workers had emerged. Colony successfully reared	
B. bimaculatus.....	23	1 queen	April 10, 1919	One queen removed on May 30, 1919. Colony successfully reared	
		1 queen	April 14, 1919		
B. bimaculatus.....	24	2 queens	April 15, 1919	One queen removed on May 12, 1919. Colony successfully reared	
B. bimaculatus.....	25	1 queen	April 16, 1919	One queen stung and killed on April 30, 1919. One removed on May 14, 1919	No interest shown in the nest
		1 queen	April 17, 1919		
B. bimaculatus.....	26	2 queens	April 21, 1919	Colony successfully started. Discontinued experiment	
B. impatiens.....	27	1 queen	April 21, 1919	Colony successfully started and reared	
B. impatiens.....	28	1 queen	April 21, 1919	*B. impatiens* dead on May 30, 1919	No interest shown in the nest
B. auricomus.....	29	2 queens	April 25, 1919	Colony successfully started and reared	
B. separatus.....	30	2 queens	May 12, 1919	One queen removed on May 15, 1919. Colony successfully started and reared	
B. separatus.....	31	1 queen	May 15, 1919	Queen removed from the nest on June 13, 1919	No interest shown in the nest
B. impatiens.....	32	1 queen	May 16, 1919	Colony successfully started and reared	
B. auricomus.....	33	1 queen	May 24, 1919	Queen died on June 13, 1919	No interest shown in the nest
B. impatiens.....	34	1 queen	May 25, 1919	Colony started by *B. impatiens* queen adopted and successfully reared by *B. auricomus* queen	
B. bimaculatus.....		1 queen	June 8, 1919		
B. auricomus.....		1 queen	June 9, 1919		
B. fervidus.....	35	2 queens	May 29, 1919	One queen removed on June 1, 1919. Other queen laid eggs, but finally deserted the nest	

TABLE I.—*Continued.*

Species.	Experiment Number.	Number of Queens and Workers.	Date Experiment Started.	Results.	Remarks.
B. separatus	36	2 queens 1 queen	May 29, 1919 June 1, 1919	One queen escaped on May 31, 1919. Another queen escaped on June 16, 1919. One queen laid eggs and partially reared larvæ, but finally deserted the nest	
B. separatus	37	2 queens	May 31, 1919	One queen *B. separatus* killed on June 12, 1919. Queen of *B. vagans* died on July 6, 1919 due to nematode infection	No interest shown in nest by any of the queens
B. separatus		1 queen	June 11, 1919		
B. vagans		1 queen	July 5, 1919		
B. separatus	38	2 queens	May 31, 1919		No interest shown in the nest
B. vagans	39	2 queens	May 31, 1919	One queen removed on July 5, 1919. Colony successfully started and reared	
B. fervidus	40	2 queens	June 12, 1919	*B. fervidus* queens all died at various times in latter part of June and July	*B. fervidus* queens made egg-cells on two different occasions, but each time deserted the comb
B. fervidus		1 queen	June 15, 1919		
P. laboriosus		1 queen	July 6, 1919		
B. fervidus	41	2 queens	June 12, 1919	One queen dead on June 14, 1919. Nest successfully started but not reared	
B. bimaculatus	42	2 queens	May 3, 1920	One queen removed on May 16, 1920. Colony successfully started	
B. bimaculatus	43	2 queens	May 3, 1920	One queen removed on May 16, 1920. Colony successfully started	
B. ternarius	44	2 queens	May 8, 1920	Queens escaped from nest on June 13, 1920	No interest shown in the nest before queens escaped
B. terricola	45	2 queens	May 30, 1920	One queen killed on June 4, 1920. Remaining queen constructed egg-cells but died on June 6, 1920	
B. terricola	46	2 queens	May 13, 1920	One queen removed on May 15, 1920. Colony successfully started	
B. perplexus	47	1 queen	May 30, 1920	Colony successfully started	
B. ternarius	48	2 queens	June 3, 1920	One queen removed on June 8, 1920. Colony successfully started	
B. impatiens	49	1 queen	May 30, 1920	Colony successfully started	
B. perplexus	50	1 queen	May 16, 1920	Colony successfully established	
B. centralis	51	2 queens	June 6, 1920	Large egg-cells made, but no eggs laid	
B. impatiens	52	1 queen	June 6, 1920	Colony successfully reared	
B. centralis	53	2 queens	June 9, 1920	Two large egg-cells made, but no eggs laid	
B. perplexus	54	1 queen	June 10, 1920	Colony successfully started	

for subsequent work along this line. In 1917, I secured four colonies out of fourteen experiments, or nearly twenty-nine per cent. Besides this, in four of the other fourteen experiments, the queens at one time or another gave evidence of some interest in the nest. In 1919, out of nineteen experiments the queens started colonies in thirteen of my artificial nests. This gave a successful percentage of seventy-seven plus in 1919. Two of the experiments of 1919 considered as failures should not really be so counted, for in one box one queen was killed and the other died of a nematode infection, and in the other instance both queens escaped. Out of thirteen experiments in 1920, ten must be considered as successful, or seventy-seven per cent. This same year, in two of the other three experiments considered as failures, the queens went so far as to make egg cells, and another colony might have been started if at that time it had been possible to continue these experiments longer in the spring. A survey of Table I. will show also that in 1917, 1919 and 1920 there were ten experiments in which but one queen was placed in each artificial nest. Colonies were started in seven of these ten experiments. The queens in two of the other three experiments of this nature never manifested any interest in the nest. One of the three, however, at one time took an interest in the nest, but finally abandoned it without laying eggs. All in all, I secured as good results, in proportion to the number of experiments, when one queen was used as when there were two.

Summary.

Summarizing, I find that it is possible to induce the queens of certain species of North American bumblebees to start colonies in confinement when either one or two queens are used in the experiments. Plath (1923) achieved similar results in 1922 by the confinement of a single queen assisted by "one to three workers." In 1919 and 1920, in starting colonies under controlled conditions an average success of over seventy per cent. was attained. These results are indicative that the style and make-up of the artificial nest used in 1919 and 1920, as well as the procedure followed, is the most likely to produce results of any method thus far described. Furthermore, for the first time normal

colonies of bumblebees were obtained by confining in an artificial nest a single queen unaided by introduced workers.

BIBLIOGRAPHY.

Armbruster, L.

1914 Probleme des Hummelstaates. Biol. Centralb., Bd. 34, pp. 685–707.

v. Buttel-Reepen, H.

1903 Die Phylogenetische Entstehung des Bienenstaates, sowie Mitteilungen zur Biologie der solitären und sozialen Apiden. Biol. Centralb., Bd. 23, No. 1, pp. 4–31. (*).

Frison, T. H.

1918 Additional Notes on the Life History of *Bombus auricomus* Robt. Ann. Ent. Soc. Amer., Vol. 10, No. 3, pp. 277–286.

Hoffer, E.

1882–83 Die Hummeln Steirmarks. Lebengeschichte und Beschreibung Derselben. II Hälfte. 32nd Jahresberichte der Steirmärkischen Landes-Oberrealschule in Graz. Pp. 1–98.

Huber, P.

1801 Observations on Several Species of the Genus *Apis*, Known by the Name of Humble-bees, and called Bombinatrices by Linnæus. Trans. of the Linn. Soc., Vol. 6, pp. 214–298.

Lepeletier, de Saint-Fargeau, Amedee.

1836 Histoire Naturelle des Insectes Hymenopteres. Tome 1, pp. 435–473.

Lindhard, E.

1912 Humlebien som Husdyr. Spredte Traek af nogle danske Humlebiarters Biologi. Tidsskr. Landbrugets Planteavl, Vol. 19, pp. 335–352, 4 figs.

Plath, O. E.

1923 Breeding Experiments with Confined *Bremus* (*Bombus*) Queens. BIOL. BULL., Vol. XLV, No. 6, pp. 325–341.

Putnam, F. W.

1864 Notes on the Habits of Some Species of Humble Bees. Proc. Essex Inst., Salem, Mass., Vol. 4, pp. 98–105.

Reaumur, M. de.

1742 Histoire des Bourdons Velus, dont les Nids sont de Mousse. Memoires pour Servir a l'Histoire des Insects. Premier Memoire, Tome 4, pp. 1–38.

Sladen, F. W. L.

1912 The Humblebee. Macmillan Co., London, pp. 283.

1912 Bumblebees and their Ways. 43d Ann. Rep. Ent. Soc. Ont., pp. 50–56.

Wagner, W.

1907 Psycho-biologische Untersuchungen an Hummeln mit Bezugnahme auf die Frage der Geselligkeit im Tierreiche. Zoologica, Bd. 19, Heft. 46, pp. 1–239.

Westerlund, A.

1898 Wie *Bombus* seinen Nestbau beginnt. Illustr. Zeitschr. Ent., Bd. 3, pp. 113–114.

STUDIES ON *PARAMECIUM*.

III. The Effects of Vital Dyes on *Paramecium caudatum*.*

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The Protozoa are in many respects more suited for a study of the action of vital dyes on protoplasm than are Metazoan tissues, since with unicellular animals it is possible to determine readily a change in the normal life of the cell as well as the exact moment of death or recovery. The protoplasm of *Paramecium* may be used as a means of studying any selectivity towards the entrance of various vital dyes, as well as the affinity of the dye for different elements in the cell. On the other hand, caution should be observed in applying results obtained in this group of animals to any other group without adequate confirmatory evidence.

Material and Methods.

The animals used belonged to a "conjugating" clone of *Paramecium caudatum* (Race 385–1, Ball, 1925), which has been maintained in culture for over four years. The dyes were obtained from the various sources indicated in Tables I. and II. Usually, the stock solutions were made up with distilled water, in such concentrations that only a very small volume of dye solution was added to a relatively large volume of culture fluid containing the *Paramecium*. However, those dyes in which the lethal concentration was only slightly less than the maximum solubility were made up in the culture medium itself, since ordinary distilled water is usually fatal to these animals. The organisms were freed from the stain by centrifuging and washing them in *Paramecium*-free hay infusion drawn from the stock cultures.

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RESULTS.

Certain dyes did not stain the cytoplasm of normal living *Paramecium* (Table I.). Of the colors tested, these were anilin blue, alizarin, erythrosin, orange G, Lyons blue, purpurin, eosin Y, trypan blue, trypan red, and congo red; all of them except Lyons blue belong to the acid group of dyes. With these stains, color became visible in the cytoplasm only when the animals were dead or moribund. Dead animals usually stained almost immediately even though the cell membrane was not broken. The macronucleus of the dead organisms was stained by all of the dyes except erythrosin and orange G. In some instances, the cytoplasm might stain before the animal was entirely dead, but such paramecia were always swollen and sluggish, and very obviously moribund. Except in purpurin or in eosin, the *Paramecium* could live normally and apparently indefinitely in any of the above dyes as long as the cytoplasm did not stain.

TABLE I.

DYES NOT STAINING CYTOPLASM OF NORMAL LIVING *Paramecium*.

Dye.	Range of Concentrations.	Toxicity at Maximum Concentration.	
		Estimated Per Cent. Dead in:	
		1 Hour.	12 Hours.
Anilin blue (N.)	1/90,000–1/55	0	5
Alizarin (M.)	1/90,000–1/16,000	0	60
Erythrosin (C. & B.)	1/90,000–1/5,000	80	100
Orange G (M.)	1/90,000–1/110	80	100
Lyons blue (C. & B.)	1/90,000–1/55	5	100
Purpurin (G.)	1/200,000–1/90,000	80	100
Eosin Y (G.)	1/90,000–1/55	100	—
Trypan blue (N.)	1/90,000–1/155	0	0
Trypan red (N.)	1/90,000–1/155	0	0
Congo red (M.)	1/90,000–1/110	3	5

(C. & B.) = Coleman and Bell Co.
(G.) = G. Grübler and Co.
(M). = H. A. Metz and Co.
(N.) = National Aniline and Chemical Co.

Purpurin, which has been used as an indicator for calcium in dead *Paramecium* (Sampson, 1925), does not stain any part of

the living animal in acid or in alkaline solution. It does produce a peculiar blistering of the pellicle, which, in the majority of the organisms, first appears at the anterior end. Somewhat later, blisters occur laterally and posteriorly, although in a few paramecia, they may arise at the posterior end before or at the same time that they appear anteriorly (cf. Child and Deviney, 1926).

Eosin did not stain the living *Paramecium* at any concentration, nor did it stain the dead organisms for several hours at a strength of 1 to 55 up to 1 to 200, even though the cell-membrane had burst and the animals were cytolized. At a greater dilution—1 to 300 or more—the cytoplasm and the macronucleus quickly took up the stain.

Trypan blue or trypan red did not stain the cytoplasm of living *Paramecium* up to a concentration of 1 to 155, practically the maximum concentration possible. These two dyes, when ingested, will color the food vacuoles but not the cytoplasm, the vacuoles destaining, however, within three hours after the organisms are removed from the dye. According to Rohde (1917), trypan red stains *Paramecium* in an acid medium, but the animals of this clone did not stain with trypan red or trypan blue either in an acid or in an alkaline solution. Becker (1926) could not stain *Opalina* in trypan blue or in trypan red 1 to 10,000 or 1 to 20,000.

Although congo red did not stain the living cytoplasm, the food vacuoles took up the stain readily, even at a dilution of 1 to 300,000.

Table II. is a record of those dyes staining living *Paramecium;* they all belong to the basic group. Of these, the most suitable are bismarck brown, methylene blue, methylene green, neutral red, and toluidin blue. Except in highly toxic concentrations, anilin yellow and methyl violet stain the cytoplasm only very lightly, the color disappearing rapidly after the animals are removed from the dye. Safranin and basic fuchsin stain the cytoplasm only at strengths which are rather highly toxic, although the latter stains the contents of the food vacuoles very deeply in non-toxic concentrations.

With the exception of bismarck brown, all of the dyes employed in sufficient concentrations to stain the cytoplasm were eventually

TABLE II.

DYES STAINING CYTOPLASM OF NORMAL LIVING *Paramecium*.

Dye.	Range of Concentrations.	Toxicity at Maximum Concentration. Per Cent. Dead in 1 Hour.	Lowest Concentration at Which Cytoplasm Stains.	Toxicity at Lowest Concentration at Which Cytoplasm Stains. Estimated Per Cent. Dead in:		Number of Hours Required for Cytoplasm to Destain.
				1 Hour.	12 Hours.	
Bismarck brown (M.)	1/750,000–1/5,000	100	1/150,000	0	5	7
Methylene blue (C. & B.)	1/1,150,000–1/5,000	100	1/100,000	5	60	7
Methylene green (C. & B.)	1/1,150,000–1/5,000	100	1/37,500	5	100	4
Neutral red (N.)	1/2,250,000–1/5,000	100	1/150,000	3	70	9
Toluidin blue (C. & B.)	1/2,250,000–1/5,000	100	1/105,000	5	70	9
Basic fuchsin (C. & B.)	1/500,000–1/5,500	100	1/25,000	30	100	9
Safranin (E. & A.)	1/100,000–1/6,600	100	1/9,000	30	100	1½
Anilin yellow (U. C.)	1/125,000–1/3,300	100	1/5,500	0	5	1
Methyl violet (B. & W.)	1/3,000,000–1/100,000	100	1/500,000	20	90	2
Janus green B (N.)	1/450,000–1/45,000	100	1/180,000	40	100	7

(B. & W.) = Burroughs Wellcome and Co.
(C. & B.) = Coleman and Bell Co.
(E. & A.) = Eimer and Amend.
(M.) = H. A. Metz and Co.
(N.) = National Aniline and Chemical Co.
(U. C.) = Chemistry Laboratory, University of California, Southern Branch.

fatal to *Paramecium*. Some of the animals were capable of living at least six days, and apparently indefinitely, in a dilute solution (1 to 125,000) of bismarck brown, the cytoplasm staining a light brown, the food vacuoles taking a deeper color. Although the macronucleus could be stained while the animal was still actively swimming (neutral red and Janus green B), in no instance was an animal so stained observed to recover.

A dye was concentrated in the food vacuoles of *Paramecium* and stained their contents at a strength very much below that required to stain the cytoplasm. After the animals were removed from the dye, the food vacuoles retained their color long after the cytoplasm had destained. This was due also, in part, to the ingestion of stained bacteria, which had been thrown down by the centrifuge and transferred along with the *Paramecium*. It was noted particularly in solutions of neutral red, where the food vacuoles continued to stain for two days after the animals had been removed from the dye to fresh infusion. Although nothing in the medium was visibly stained, the contents of the food vacuoles took on a pink color as the latter formed at the base of the gullet, and became a deep red as the vacuole began passing forward from the posterior end of the animal. Any observation of the time required for destaining *Paramecium* should be based on the behavior of the cytoplasm and not of the food vacuoles, which may retain their color until nearly all of the bacteria in the medium have been digested.

With all of the stains used, the color of the cytoplasm disappeared fairly rapidly after the organisms were removed from the dye solution, in no case being detectable for more than nine hours, and in many instances fading out much sooner. The time required for destaining the cytoplasm was independent both of the length of time it had been stained and of the concentration of the dye used. If the stained animals were removed to a dilute solution of the dye, their life was prolonged, but they were eventually killed unless the cytoplasm destained. It is possible that the results of Baldwin (1920), in which paramecia so treated lived for more than eight days may have been based on the retention of the dye in the food vacuoles rather than in the cytoplasm proper.

The comparatively rapid loss of the color by stained paramecia indicates the ability of the protoplasm of *Paramecium* to reduce or to eliminate the dye in a few hours; or perhaps even marks a complete change of protoplasm in this time.

A solution of 1 to 180,000 Janus green B shows almost immediately a number of small rod-like bodies, probably mitochondria (Causey, 1926; Hogue, 1926). These were somewhat more widely scattered through the cytoplasm than those shown in Causey's figures, although they could also be detected between and around the food vacuoles. The dye is exceedingly toxic for *Paramecium*, killing them in a few hours at a concentration as low as 1 to 450,000. The cytoplasm stains bluish green at 1 to 180,000, the macronucleus taking on a yellow color before the animal dies. The food vacuoles are at first bluish green, later becoming light pink due to the reduction of the Janus green B to diethylsafranin.

All of the above experiments were performed in the diffuse light of the laboratory, the cultures being kept under glass, out of the direct sunlight. Animals stained with certain dyes (Table III.) give a very striking reaction to strong light. These stained *Paramecium* drawn from a flourishing culture soon become sluggish and settle practically motionless on the bottom of the Syracuse watch glass. They can be observed in this condition under a compound microscope with the iris diaphragm almost completely closed, and a heat screen interposed between the organisms and the source of light. If the diaphragm now be suddenly opened, practically all of the animals begin darting violently about, giving the avoiding reaction and soon swimming out of the field. The reaction begins in from four to five seconds after opening the diaphragm, and occurs almost simultaneously in 90 to 95 per cent. of the animals exposed. If the *Paramecium* are observed under a 16 mm. objective, the field will be entirely empty in two to three minutes, even though several hundred motionless animals may have been present previously. If the diaphragm be closed before the field is completely vacant, the remaining organisms settle to the bottom of the dish again within one minute. If only the food vacuoles are stained but not the cytoplasm, the animals do not exhibit these reactions to

light. The uniformity in reaction-time is evidently due to the fact all the *Paramecium* belonged to a single clone.

TABLE III.

EFFECT OF LIGHT ON TOXICITY OF DYES FOR *Paramecium*.

Strength of Dye Used.	Number of Hours Required to Kill in:		Light Reaction.
	Strong Light.	Darkness.	
Eosin Y (G.) 1/250.....	19 (95% dead in 3 hours)	Lives indefinitely	+
Methylene blue (C. & B.) 1/100,000	1	24	+
Methylene green (C. & B.) 1/45,000	2	17	+
Neutral red (N.) 1/125,000.....	1	25	+
Toluidin blue (C. & B.) 1/107,000..	1	48	+
Bismarck brown (M.) 1/125,000....	Lives indefinitely	Lives indefinitely	−
Basic fuchsin (C. & B.) 1/18,000...	2½	2½	−
Trypan blue (N.) 1/1,500.....	Lives indefinitely	Lives indefinitely	−
Trypan red (N.) 1/1,500.....	Lives indefinitely	Lives indefinitely	−

Furthermore, those stained paramecia exhibiting this light reaction die much sooner when exposed to strong light than when kept in the dark. Table III. shows the difference in survival-time between the two groups of organisms. An ordinary Mazda lamp served as the source of light. The dishes containing the organisms were covered with water-filled glass containers and exposed to a current of air from an electric fan. The temperature in the two series did not differ by as much as a degree during the course of the experiments. Animals stained in bismarck brown, basic fuchsin, trypan blue, or trypan red showed no change in behavior upon sudden illumination; correspondingly, the toxicity of these dyes was not increased in the light. While living *Paramecium* did not stain in eosin, and although a concentration as great as 1 to 250 was not toxic for them as long as they were kept in the dark, they had a marked avoiding reaction to light and died rapidly under strong illumination. Raab (1900) studied the greater toxicity of *fluorescent* dyes for infusoria, and similar results were recorded by Pereira (1925), who tested the action of eosin on the germ cells and larvæ of *Arbacia*.

According to Packard (1925), light increases the permeability of the protoplasm of *Paramecium;* the greater toxicity is not solely a matter of permeability, however, since such stains as neutral red penetrate the cytoplasm almost immediately, yet the animals live twenty times as long in the dark as under strong illumination. Child and Deviney (1926) noted greater toxicity of eosin, neutral red, and methylene blue for *Paramecium* upon exposure to light, while Baldwin (1920) found that stained paramecia were more susceptible to X-rays than unstained ones. In the clone used in the present experiments, long contact with the dye does not produce any acclimatization on the part of the *Paramecium;* animals kept in the dark in neutral red 1 to 125,000 or in methylene blue 1 to 100,000 for eighteen hours die just as soon under strong illumination as those exposed to the light and the stain simultaneously. Furthermore, merely subjecting the dye to intense light does not make it more toxic when subsequently added to cultures of *Paramecium.*

Discussion.

The literature on vital staining in the Protozoa has been well reviewed by Nirenstein (1920) and by Vonwiller (1921), and consequently will not be considered here.

All of the dyes which stained the cytoplasm of normal living *Paramecium* belonged to the basic group. None of the acid dyes stained the cytoplasm of normal animals, although most of them stained the dead or dying organisms. Lyons blue was the only basic dye not staining the living animals. There have been many different interpretations of the meaning of the reactions taking place between different dyes and living protoplasm (Nirenstein, 1920). If it be concluded from the above results that, under normal conditions, the protoplasm of *Paramecium* is acid in reaction, this would confirm Crozier's observation (1923), using brom thymol blue as an indicator, that the pH of the living protoplasm of *Paramecium* is 6.7 or less, and not alkaline or neutral as is usually maintained.

The period required for destaining the organisms was independent both of the length of time necessary for staining them, and of the concentration of the dye used. Furthermore,

if the cytoplasm stained at all, the color was as intense with a dilute as with a concentrated solution of the stain. In dilute solutions, the cytoplasm or the food vacuoles might stain deeply, yet the fluid medium or the bacteria might show only a very slight color. According to Nirenstein (1920), the capacity of the living cell for taking up dyes depends upon the distribution of lipoids in the protoplasm. It may possibly mark merely a more intense staining reaction rather than an increased concentration of the dye. The failure of the acid dyes to stain the cytoplasm does not prove that they fail to enter as freely as do the basic ones; the behavior of the organisms indicates rather that the dyes of the acid group do penetrate the cell without staining the cytoplasm.

Certain dyes act as sensitizers for the destructive action of light; their toxicity is not increased by exposure to strong light before addition to the *Paramecium* cultures. The effect is not solely one of greater permeability since the shorter length of life in light bears no relation to the ease of penetration of the dye. With this clone of *Paramecium*, the harmful effect of light for the stained organisms began within five seconds of exposure, as indicated by the behavior of the animals themselves.

This work on vital stains was started originally in the hope of employing it to mark individuals belonging to different clones of *Paramecium*. Thus, it would be possible to determine whether or not extra-clonal conjugation can be obtained under experimental conditions. However, the rapidity with which the cytoplasm of these animals loses its color after being removed from the stain precludes the use of this method as a means of identification. It seems probable that we shall have to employ morphological differences, other than those of size, such as the notched mutant recently described by Dawson (1926).

Conclusions.

1. The only dyes staining the cytoplasm of normal living *Paramecium* belonged to the basic group. Those found to be most suitable were bismarck brown, methylene blue, methylene green, neutral red, and toluidin blue.

2. The cytoplasm of normal animals could not be stained by

any of the acid dyes used, although these might stain the contents of the food vacuoles, or the cytoplasm of dead or dying *Paramecium*.

3. Animals with stained cytoplasm could live indefinitely in a dilute solution of bismarck brown; in all of the other dyes used, the stained paramecia eventually died unless they were removed from the stain. The presence of the dye in the food vacuoles but not in the cytoplasm was not necessarily fatal to *Paramecium*.

4. Although the macronucleus could be stained while the animal was still alive, such animals eventually died even though removed from the stain.

5. The stain disappeared from the cytoplasm of the living animals within a few hours after their removal from the dye solution.

6. The exposure to light of *Paramecium* having the cytoplasm stained by certain dyes, or of *Paramecium* in a concentrated solution of eosin, produced a marked avoiding reaction within five seconds. The animals giving this reaction died much more rapidly in strong light than in darkness.

BIBLIOGRAPHY.

Baldwin, W. M.

'20 A Study of the Combined Action of X-rays and of Vital Stains upon Paramœcia. BIOL. BULL., *39*, 59–66.

Ball, G. H.

'25 Studies on *Paramecium*. I. II. Univ. Calif. Publ. Zoöl., *26*, 353–433.

Becker, E. R.

'26 Vital Staining and Reduction of Vital Stains by Protozoa. BIOL. BULL., *50*, 235–238.

Causey, D.

'26 Mitochondria in Ciliates with Especial Reference to *Paramecium caudatum* Ehr. Univ. Calif. Publ. Zoöl., *28*, 231–250, pls. 22–24.

Child, C. M., and Deviney, E.

'26 Contributions to the Physiology of *Paramecium caudatum*. Jour. Exp. Zoöl., *43*, 257–312.

Crozier, W. J.

'23 A Note on the Reaction of Protoplasm. Proc. Soc. Exp. Biol. and Med., *21*, 58.

Dawson, J. A.

'26 A Mutation in *Paramecium aurelia*. Jour. Exp. Zoöl., *44*, 133–157, 1 pl.

Hogue, M. J.

'26 Staining Protozoa with Janus Green B. Stain Technology, *1*, 35–36.

Nirenstein, E.

'20 Über das Wesen der Vitalfärbung. Arch. ges. Physiol., *179*, 233–337, 1 pl.

Packard, C.

'25 The Effect of Light on the Permeability of *Paramecium*. Jour. Gen. Physiol., *7*, 363–372.

Pereira, J. R.

'25 On the Combined Toxic Action of Light and Eosin. Jour. Exp. Zoöl., *42*, 257–262.

Raab, O.

'00 Über die Wirkung fluorescirender Stoffe auf Infusorien. (Munich, Oldenbourg), Inaug. Diss. Munich, 27 pp.

Rohde, K.

'17 Untersuchungen über den Einfluss der freien H-ionen im Inneren lebender Zellen auf den Vorgang der vitalen Färbung. Arch. ges. Physiol., *168*, 411–433, pls. 2–3.

Sampson, M. M.

'25 Conditions of Validity of Macallum's Microchemical Test for Calcium. Science, *62*, 400–401.

Vonwiller, P.

'21 Intravitale Färbung von Protozoen. Abderhalden's Hdbch. der biolog. Arbeitsmethoden, Abt. V,. no. 2, 87–96.